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Helminths as an alternative therapy for intestinal diseases

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Abstract

Animal models and clinical studies have shown that helminth infections exert immunomodulatory activity, altering intestinal permeability and providing a potential beneficial action on autoimmune and inflammatory disorders in human beings, such as inflammatory bowel disease (IBD) and celiac disease. This is consistent with the theory that intestinal microbiota is responsible for shaping human immunological responses. With the arrival of the immunobiologic era and the use of antibodies, we propose a distinctive pathway for treating patients with IBD and celiac disease. We have some evidence about the safety and tolerability of helminth use, but evidence about their impact on disease activity is lacking. Using worms to treat diseases could be a possible way to lower treatment costs, since the era of immunobiologic agents is responsible for a significant rise in expenses. Some questions remain to be investigated regarding the use of helminths in intestinal disease, such as the importance of the specific species of helminths used, appropriate dosing regimens, optimal timing of treatment, the role of host genetics, diet, environment, and the elucidation of the exact mechanisms of action. One promising approach is the use of helminth-derived anti-inflammatory molecules as drugs. Yet there are still many challenges with this method, especially with regard to safety. Studies on intestinal permeability point to *Strongyloides stercoralis* as a useful nematode for these purposes.

Key words: Helminths; Strongyloidiasis; Immunology; Inflammation; Inflammatory bowel diseases; Intestinal diseases; Intestinal permeability; Celiac disease

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Core tip: Inflammatory bowel disease and celiac disease are immune-mediated pathologies that remain

a treatment challenge for gastroenterologists. Despite the recent introduction of novel therapies, notably biological agents and newer management strategies, there are still many patients who do not respond, or have a poor response to current treatments. Helminth therapy seems a promising pathway to newer drugs, because it has been proven to alter intestinal permeability, altering the host's immune response to a Type 2 cytokine-mediated response in animal models and pre-clinical studies. This editorial aims to stimulate further research in this field, hoping for better care for our patients.

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INTRODUCTION

The intestinal epithelium functions as an important part of the digestion and absorption of fluids and nutrients. It also plays an immunologic role because it protects the host from environmental pathogens and antigens^[1]. When this barrier is altered, it leads to ease of antigen entry and subsequently to immune stimulation and inflammation^[2].

Many intestinal diseases have immunogenic components that alter intestinal permeability. Some pathologic processes increase intestinal permeability, such as inflammatory bowel disease (IBD)^[3-11], atopic eczema^[12], celiac disease, dermatitis herpetiformis^[13], cystic fibrosis^[14,15], alcohol consumption^[16], use of nonsteroidal anti-inflammatory drugs^[17-20], and acute infectious diarrhea^[12,21]. In contrast, some infections, such as those caused by *Blastocystis hominis*^[22], can decrease intestinal permeability and alter the barrier function of the epithelium. These may thus provide an alternative pathway for treating patients by deviating the host's immunologic response.

Intestinal permeability can be measured in many ways. One is *via* measuring urinary clearance of radioactive chromium-51 labeled ethylenediaminetetraacetic acid (⁵¹Cr-EDTA). When urinary clearance of ⁵¹Cr-EDTA is decreased, this indicates decreased intestinal permeability. Werneck-Silva *et al*^[23] demonstrated that infection with *Strongyloides stercoralis* can diminish intestinal permeability compared to healthy volunteers (*P* = 0.0001). Intestinal infection with *S. stercoralis* led to abnormalities in mucus secretion and intestinal motility, as well as possible loss of macromolecules. *S. stercoralis* is a soil-transmitted helminth and is one of the most common parasites that affects patients

living in tropical areas^[24]. It infects 100 to 200 million people worldwide^[25]. It predominantly compromises the mucosa of the duodenum and upper jejunum, although the whole intestinal wall or more extensive segments of the intestine can be involved, especially in immunocompromised patients^[26-28].

DISCUSSION

Hygiene hypothesis

The hygiene hypothesis was initially described in the 1970s. It suggests that the higher incidence of allergic diseases in predominantly urban white communities, compared to those rural and indigenous, is due to the less frequent viral, bacterial and helminth infections^[29]. A decade later, Strachan proposed that reduced exposure to infections in early childhood, owing to a combination of diminished family size, improved living standards and higher levels of personal hygiene, might result in an increased risk of allergic disease later in life^[30]. In addition to allergic disease^[31-33], it is believed that the recent increase in other autoimmune and inflammatory disorders, especially in developed countries, could be explained by a similar hypothesis. Many factors may be involved, such as changes in intestinal microbiota during childhood^[34].

Helminth infections, in specific intestinal worms, are a particular area of research interest, since they can modulate the host response, inducing immunologic tolerance. Aoyama *et al*^[35] have demonstrated an inverse relationship between autoimmune liver diseases, such as primary biliary cirrhosis, autoimmune hepatitis, and primary sclerosing cholangitis, and *S. stercoralis* infection. Recent studies point to the negative effects of deworming, since the helminths are able to not only downregulate specific immune responses, but also to modulate autoimmune and allergic inflammatory responses, contributing to metabolic homeostasis^[36]. The study on the use of helminths and their products as anti-inflammatory treatments is a growing field.

Helminth-induced immune responses

Parasitic helminths evolved with the mammalian immune system, promoting their own survival by altering host immune responses^[37]. The immune response induced by these worms is dependent on a Type 2 cytokine response, involving the secretion of interleukin (IL)-4, IL-5, IL-9 and IL-13, accompanied by the activation of intestinal mast cells^[38], eosinophils, goblet cells, enterocyte proliferation and intestinal contractility^[39]. Granuloma formation then occurs, isolating the eggs and larvae, and inducing tissue repair^[40]. Other accessory pathways are activated, including the upregulation of regulatory T cell and IL-10 and/or transforming growth factor beta levels, leading

to a predominantly anti-inflammatory response. It has been shown that IL-10- deficient-mice with helminth infections have higher mortality and/or morbidity^[41].

The role of CD4+ T cells in expressing Th1, Th2 and Th17 cytokines in human infection with *S. stercoralis* is better explored by Anuradha *et al.*^[42], who demonstrated a decrease in functional Th1 and Th17 cells and an increase in functional Th2 cells, compared to uninfected individuals. The regulation of Th1, Th2 and Th17 cells was predominantly dependent on IL-10, while the regulation of Th2 but not Th1 or Th17 cells was also dependent on TGF β . Anuradha *et al.*^[43] also examined the circulating levels of cytokines in infected individuals ($n = 32$) compared to those uninfected, discovering significantly lower circulating levels of pro-inflammatory cytokines (gamma interferon, tumor necrosis factor alpha and IL-1) and significantly higher levels of anti-inflammatory cytokines (IL-4, IL-5, IL-9, IL-10, IL-13, IL-27, IL-37, and TGF- β). In addition, treatment of infection led to an opposite immunological response in the two studies. The question is whether these anti-inflammatory properties could be used in intestinal disorders with a predominant Type 1 cytokine response.

Helminth therapy for intestinal inflammation

The ability of helminth infections to alter and/or to suppress immune responses and intestinal inflammation could be useful in IBD^[37]. To date, only two species of helminths have been used as clinical treatment: *Trichuris suis*, the pig whipworm, and *Necator americanus*, the human hookworm.

The first is acquired by ingestion of ova and colonization of the caecum and proximal colon of the human gut by worms, which only lasts a few weeks. The second infection develops after percutaneous administration of larvae that migrate to the small intestine, where they survive by feeding on blood from the mucosa. *T. suis*, due to the species-specificity and the lack of chronic infection, requires repeated treatments, although it poses lesser health issues. In the case of *N. americanus*, the long lasting infection means greater risk of anemia and gastrointestinal symptoms, which could be deleterious side effects^[37]. To date, there are no studies of *S. stercoralis* for treating intestinal inflammation.

Evidence of helminth therapy in inflammatory bowel disease

Approximately 15 years ago, the first clinical studies of helminth therapy for intestinal disease in humans utilized embryonated viable eggs of *T. suis* in the treatment of ulcerative colitis (UC) and Crohn's disease (CD). These studies showed safety, tolerance and a significant disease remission when oral administration of viable and embryonated eggs was performed repeatedly^[44,45]. A placebo-controlled, double-blind,

randomized trial in UC patients significantly improved the disease activity index and showed no side effects, although the remission rate was not different than placebo^[46]. Another double-blind, placebo-controlled, randomized study (NCT01434693) reported that a single dose of *T. suis* ova (TSO) up to 7500 ova was well tolerated and did not result in short- or long-term treatment-related side effects in CD patients^[47].

A brief review of Clinicaltrials.gov reveals three interventional studies of TSO in CD and two in UC. In CD, the studies were sponsored by Coronado Biosciences, which changed its name to Fortress Biotech, and by Dr. Falk Pharma GmbH. TRUST-1 (NCT01576471), a Phase 2 clinical trial evaluating 250 North American patients with moderate-to-severe disease did not improve the disease activity index or remission rates, although a nonsignificant improvement was noted in patients with a more severe disease score.

TRUST-2 (NCT01279577), a double-blind, placebo-controlled, randomized trial of 252 European adults with mildly-to-moderately active ileo-colonic, uncomplicated CD, documented that the administration of fortnightly doses of 250, 2500, or 7500 TSO/15 mL suspension/day over 12 wk, with a four-week follow-up, was safe, with no serious adverse drug reactions. There was a dose-dependent immunological response, but no TSO dose showed a clinically relevant effect over placebo for the induction of clinical remission (CD Activity Index < 150) or response^[48].

In UC, the first study was sponsored by the New York University School of Medicine, and the second by the National Institute of Allergy and Infectious Diseases. Both were terminated due to a small sample size and because it was not possible to draw meaningful conclusions. MUCUS (NCT01433471), a randomized, double-blind, placebo-controlled crossover study, was conceived to examine mucosal immunity after therapy with 2500 eggs by mouth every 2 wk for 12 wk. Primary outcomes were designed to better understand the mechanism of action of TSO on the intestinal mucosa and secondary outcomes were to bring about changes in the Mayo Score and in the Simple Clinical Colitis Activity Index.

A second, more controversial approach, was the use of *N. americanus*. Croese *et al.*^[49] showed that 7 of 9 patients with CD infected with 25-50 larvae followed over 20 wk experienced an improved CD activity index, while the other 2 worsened. There were no search results for interventional studies regarding the use of *Strongyloides*, *Ascaris*, *Ancylostoma*, *Wuchereria*, *Onchocerca*, *Toxocara* or *Enterobius* in CD or UC on Clinicaltrials.gov.

Evidence of helminth therapy in celiac disease

There are few studies examining the use of helminths

in celiac disease, most of which with small samples. McSorley *et al.*^[50] and Daveson *et al.*^[51] examined 20 celiac patients followed by wheat challenge after 20 wk exposed to 5-10 larvae of *N. americanus*, compared to placebo, at Princess Alexandra Hospital, in Brisbane, Australia. The dose was well tolerated and analysis showed reduced gamma interferon and interleukin-17A in duodenal biopsies. No difference in symptoms was observed.

Another Australian clinical trial, NaCeD study (NCT016619330), evaluated the desensitization and gluten tolerance of 12 diet-managed celiac patients. They were previously infected with *N. americanus* and exposed to small incremental doses of gluten, in the form of pasta, over 12 wk. The mucosal histopathology before and after gluten challenge was examined. There were no significant differences in terms of duodenal villus height and crypt depth ratio and intraepithelial lymphocyte count.

Another clinical trial (NCT02754609) was registered in 2016 by James Cook University in Queensland, Australia, on Clinicaltrials.gov. This trial aims to be a phase 1b multicenter, multinational, randomized, double-blind, placebo-controlled clinical trial with a single-blind arm and an open label extension phase. The objective is to evaluate the safety and predictability of escalating gluten consumption to activate celiac disease. The cohort with diet-managed disease will be treated with placebo or with low- and medium-dose hookworm inocula. The primary outcome is to measure the difference in duodenal villus height and crypt depth ratio between baseline (week 2) and week 42.

There were no search results for interventional studies about *Trichuris*, *Strongyloides*, *Ascaris*, *Ancylostoma*, *Wuchereria*, *Onchocerca*, *Toxocara* or *Enterobius* use in the treatment of celiac disease on Clinicaltrials.gov.

Other uses for helminth therapy

There are other studies examining the role of helminth therapy in allergy, atopy and asthma, with conflicting results. The biggest problem seems to be that these studies proved that preventing allergic reactivity is possible, but only a handful have reported the ability to impact an already established process. In addition to allergy, a number of clinical trials are currently registered for the use of TSO in patients with multiple sclerosis^[52], psoriasis, autism and rheumatoid arthritis^[37], and for the use of *N. americanus* in patients with multiple sclerosis^[35].

Helminth products as possible new drugs

Helminths are complex organisms that have a variety of immunomodulatory substances, such as lipids, carbohydrates and proteins, and jointly defined excretory-secretory products (ES). The identification

of helminth products that can be used as biologicals in place of whole parasites is an engaging area of research. The ES-62 glycoprotein from the filarial nematode *Acanthocheilonema vitae* is one of the most studied compounds and is capable of promoting a Th2 response, inhibiting Th1 and Th17. Animal studies have demonstrated the ability of various ES products to inhibit intestinal inflammation in colitis models. These studies suggest a potential way for discovering new drugs for IBD. Concerns about antigenicity and safety need to be clarified prior to clinical testing^[53].

Most published studies focus on the use of nematodes and their products in the treatment of intestinal disease. Unlike most studies, there is an ongoing multicenter phase 2 clinical trial, in the recruiting phase, sponsored by the University Hospital, Lille, France, named ACROHNEM (NCT02281916). It is designed to assess safety and tolerability of P28GST (protein 28 Kd glutathion S transferase), aiming to control inflammation in moderate CD, before or after intestinal resection surgery. P28GST is a parasite enzyme molecule from *Schistosoma* with potent immunogenic and anti-oxidant properties. Based on the experimental evidence of its anti-inflammatory properties, investigators hypothesized that the administration of P28GST could protect against recurrence after intestinal resection surgery in CD. To carry out this study, 24 moderate CD patients will be enrolled. Patients with moderate CD will be included after intestinal resection surgery. Drug therapy will consist of three injections of 100 µg of P28GST for 3 mo (one injection per month). The main objective of this study is to assess safety and tolerability in a 1-year follow-up. Secondary objectives are to control immunologic and inflammatory blood and tissue markers and evaluate clinical recurrence as assessed by CDAI (CD Activity Index).

CONCLUSION

The intestinal microbiota is responsible for shaping the human immune system, and the composition of the microbiome can alter and deviate specific host immune responses. Although much has been written about bacteria, we cannot forget that other organisms, such as the helminths, may possibly play an important role in maintaining a "healthy intestinal community"^[54].

Mouse models^[55] and human cross-sectional studies have shown that chronic helminth infections exert immunomodulatory activity and are able to regulate the host immune response, providing a potential beneficial action on autoimmune and inflammatory disorders in humans, such as IBD, celiac disease, asthma, atopy, allergy, multiple sclerosis, psoriasis, autism and rheumatoid arthritis^[37].

We have some evidence about the safety and

tolerance of helminth use, but evidence about the impact on various intestinal diseases is lacking. We need more clinical studies with larger samples, longer follow-ups and standardized doses of helminths and helminth products. Some questions remain to be investigated regarding the use of helminths in intestinal disease, such as the importance of the particular species of helminths used; appropriate dosing regimens (low or high); optimal timing of treatment (before the onset of disease, in acute or chronic disease, or at younger ages); the role of host genetics, diet and environment, and elucidation of the exact mechanisms of protective effect.

In regard to the species of helminth, we believe that the majority of the studies had negative results because of the use of *T. suis*. This pig whipworm induces a less intense and persistent inflammatory response, although Williams *et al.*^[56] verified that *T. suis* can mature to adult size and reproduce in humans. That is why we see *S. stercoralis* as a more potentially useful nematode, as it has proven to significantly diminish the intestinal permeability in humans^[23], altering the interleukin profile in a more systemic way^[42]. The prolonged interaction between *S. stercoralis* and its host induces a greater immunomodulatory action. Regarding the appropriate dose and duration of treatment, we have little comprehension of how much and how long is required to exert a significant and beneficial effect; therefore, safety concerns limit the dose that can be applied.

One important challenge is the high polymorphism of the human species, which reacts in a spectral manner to helminth infection. The genetic profile of each individual alters this response. In this context, the identification of helminth-derived anti-inflammatory molecular mediators may be a better and promising approach, since it replicates the benefits without the detriments^[57]. There are many challenges with this method, such as the selection of a substance with a good safety profile and low antigenicity that is easily produced and that has a significant impact on clinical trials.

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Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis

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Abstract

Inflammatory bowel diseases (IBD), including Crohn's disease and ulcerative colitis, are complex diseases that result from the chronic dysregulated immune response in the gastrointestinal tract. The exact etiology is not fully understood, but it is accepted that it occurs when an inappropriate aggressive inflammatory response in a genetically susceptible host due to inciting environmental factors occurs. To investigate the pathogenesis and etiology of human IBD, various animal models of IBD have been developed that provided indispensable insights into the histopathological and morphological changes as well as factors associated with the pathogenesis of IBD and evaluation of therapeutic options in the last few decades. The most widely used experimental model employs dextran sodium sulfate (DSS) to induce epithelial damage. The DSS colitis model in IBD research has advantages over other various chemically induced experimental models due to its rapidity, simplicity, reproducibility and controllability. In this manuscript, we review the newer publicized advances of research in murine colitis models that focus upon the disruption of the barrier function of the intestine, effects of mucin on the development of colitis, alterations found in microbial balance and resultant changes in the metabolome specifically in

the DSS colitis murine model and its relation to the pathogenesis of IBD.

Key words: Dextran sodium sulfate; Experimental colitis; Inflammatory bowel disease; Pathogenesis; Intestinal barrier

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Core tip: In the last few decades the proliferation of research in experimental colitis models of inflammatory bowel diseases (IBD) has had profound effects in our understanding of human IBD pathophysiology as well as to exploit potential therapeutic avenues outside of immunologic therapy. The dextran sodium sulfate colitis model, through its rapidity, simplicity, reproducibility and controllability has been instrumental in our understanding of intestinal barrier function through the dysregulation of mucin, interaction with the intestinal microbiome and metabolome.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a group of inflammatory conditions of the colon and small intestine caused by a dysregulated immune response. Crohn's disease (CD) and ulcerative colitis (UC) are the principal types of IBD. Both usually involve severe diarrhea, pain, fatigue and weight loss. IBD can be debilitating and sometimes leads to life-threatening complications. A convenient and time honored approach to study the pathogenesis and complexity of human IBD has been the development of a variety of animal models. These animal models have provided meaningful and indispensable insights into the histopathological and morphological changes in the intestinal tract related to the pathogenesis of human IBD^[1]. While no single model has proven to sufficiently represent the complexity of the clinical and histopathological characteristics of human disease, the collective data obtained from these various animal models have provided a more detailed understanding of the underlying principles of human IBD pathogenesis^[2,3]. These models have become an indispensable tool to elucidate the histopathological, immunological and morphological changes in the intestinal tract and potential therapeutic targets. These various models can be grouped into categories broadly defined as spontaneous colitis, chemically inducible

colitis, genetically modified and adoptive transfer models^[1,4-7].

The most widely used mouse model of colitis employs dextran sodium sulfate (DSS), a chemical colitogen with anticoagulant properties, to induce epithelial damage. The DSS colitis model lends itself to IBD research due to its rapidity, simplicity, reproducibility and controllability. Acute, chronic and relapsing models of intestinal inflammation can be achieved by modifying the concentration of DSS and the frequency of administration^[8]. There are excellent and exhaustive reviews focused on the immunological aspects of experimental animal models in inflammatory bowel disease and we would recommend the reader to refer to these articles for the specifics in immunology^[2,9]. In this review, we aim to provide an updated and concise review of the less publicized aspects of research in murine colitis models that focus upon the barrier function of the intestine in one specific chemically induced experimental colitis model, the DSS colitis murine model.

HUMAN IBD PATHOPHYSIOLOGY: MECHANISTIC INSIGHTS

IBD is a disorder of chronic intestinal inflammation without an exact etiology. The leading hypothesis on IBD pathogenesis states that it is an inappropriate and overly aggressive inflammatory response to enteric microbes in a genetically susceptible host with environmental factors precipitating the onset or reactivation of disease^[10,11]. Epidemiologic data suggest these environmental factors include antibiotic use, microbial exposure and possibly dietary components^[12-15]. Host genetics, luminal microbiome and its associated antigens and immune response, all have been implicated in playing important roles in IBD pathogenesis (Figure 1).

Genetics

Advances in IBD genetics have indicated modifications in genes regulating mucosal barrier integrity and function, innate immune response and microbial homeostasis^[11]. Thus far, four genes have been associated with increased susceptibility to CD and one gene mutation has been associated with ulcerative colitis^[11]. The most widely known and studied gene implicated in IBD is the gene for *CARD15*, formerly known as *NOD2*, which is responsible for luminal bacterial recognition. The leucine-rich repeat region of *CARD15* binds muramyl dipeptide (MDP), which is the active moiety of peptidoglycan. This binding of bacterial peptidoglycan to MDP activates NF- κ B and mitogen-activated protein kinase signaling pathways, causing the production of various cytokines including TNF and IL-1 β ^[10,16,17]. In normal circumstances, the pro-inflammatory cytokine secretion by intestinal APCs is minimal, yet bacterial killing occurs. This indicates that the intestinal immune

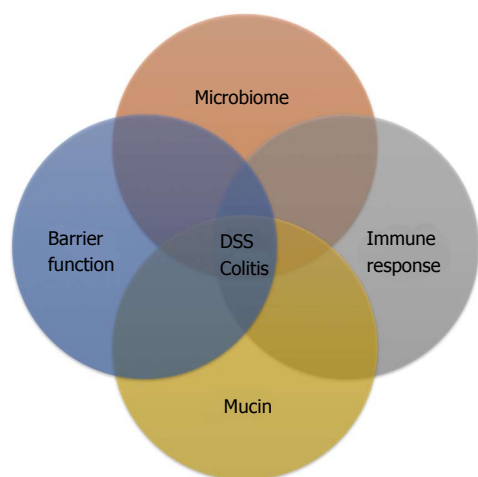


Figure 1 Factors that play important roles in the pathogenesis of inflammatory bowel diseases. DSS: Dextran sodium sulfate.

system can defend against luminal microbiota without appreciable tissue injury^[10]. The importance of NF κ B activation in the clearance of invasive bacteria is underscored by the observations that *CARD15* gene mutation fails to clear *Salmonella* from epithelial cells leading to increased bacterial interaction with the host's luminal defense mechanisms. Indeed, heterozygosity for a *NOD2* polymorphism confers a 1.75 to 4-fold increased risk whereas homozygosity confers even a much higher risk (11 to 27-fold) for development of IBD^[10,18].

Microbiome and dysbiosis

A complex network of interactions exists between the host intestinal epithelial cells (IEC), host immune cells and the abundant intestinal microbiota. An altered balance of commensal pathogenic microbiota could lead to a pro-inflammatory milieu that exacerbates intestinal inflammation^[11]. The dysbiosis theory suggests that intestinal microbiota of IBD patients change in diversity, composition and localization compared to that of healthy controls^[19-24]. Fecal microbiota studies on IBD patients have revealed a decreased frequency in Bacteroidetes and Firmicutes and an increase of Proteobacteria and Actinobacteria phyla^[21,23-26]. Advances in metagenomics sequencing of microbial RNA have confirmed decreases in bacterial composition and diversity in IBD patients compared to normal population^[15]. Likewise, defects in the intestinal epithelial barrier integrity suggests increased uptake of luminal antigens that further leads to persistent immune activation^[11]. Evidence suggests that reduced mucin production, through goblet cell depletion and epithelial cell tight junction dysfunction, by facilitating the access of gut luminal antigens to the intestinal mucosa, are also involved in the pathophysiology of IBD^[15]. Additionally, *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum and one of the most abundant species in the healthy human colon, is

underrepresented in IBD patients and altered in terms of disease activity specifically in CD patients^[19,20,24,27-30].

It is still uncertain as to whether the dysbiosis is the primary cause of inflammation or merely a secondary phenomenon of IBD. The uncertainty stems from the fact that intestinal microbiota analysis in IBD patients is performed after the development of the disease rather than prior to development^[22]. However, the pathogenic role of the microbiome in IBD pathogenesis and therapy is indicated by studies showing an improvement in IBD by antibiotic treatment^[31,32]. Indeed, treatment of UC patients with antibiotics improves mucosal inflammation^[15]. Further, remission of inflammation and mucosal healing was observed in CD patient's that underwent diversion of feces with subsequent disease reactivation after infusion of feces^[33]. Likewise, defects in the intestinal epithelial barrier integrity suggests increased uptake of luminal antigens that further leads to persistent immune activation^[11]. Evidence suggests that reduced mucin production, through goblet cell depletion and epithelial cell tight junction dysfunction, by facilitating the access of gut luminal antigens to the intestinal mucosa, are also involved in the pathophysiology of IBD^[15]. Collectively, these observations attest to the role of microbial dysbiosis in the induction of IBD^[34].

Immune response

The innate and adaptive immune system activation and dysfunction as well as a loss of tolerance to enteric commensal bacteria contribute to the abnormal inflammatory response in the intestinal tract in patients with IBD^[11,35]. Characteristic histological findings in IBD is an influx of innate immune cells (neutrophils, macrophages, dendritic cells and NK cells) as well as adaptive immune cells (B cells and T cells) into the lamina propria. With activation of immune cells, there is an elevation in the TNF- α , IL-1 β , IFN- γ and cytokines levels. Recent advances in genome-wide associated studies and immunological studies suggest aberration in the mucosal innate response, innate microbial sensing, autophagy and unfolded protein response are potential pathogenic pathways in IBD^[35].

Microbial antigen sensing by the innate immune system is mediated by pattern recognition receptors (PRR), such as Toll-like receptors (TLR) that recognize pathogen associated molecular patterns. The stimulation of PRRs results in a signaling cascade in which NF κ B activation produces pro-inflammatory mediators, thereby ensuring an effective and appropriate innate response to presented antigens. Specific TLR that have been implicated in CD include nucleotide-binding oligomerization domain (NOD)-like receptors, specifically *NOD2*, which encodes an intracellular sensor and is stimulated by specific components of bacterial peptidoglycan that results in NF κ B activation as mentioned in the earlier section on genetics^[35].

Adhesion molecules, such as intercellular cell adhesion molecule 1 (ICAM1) are necessary for the circulating cells to adhere to the activated endothelium, which leads to extravasation of mononuclear and polymorphonuclear cells into the inflammatory tissue^[11]. In addition, adhesion molecules mediate migration of the extravasated immune cells through the stroma to the source of maximal chemokine production, as well as through the epithelium to the lumen, where they produce crypt abscesses^[11]. Historically, the NF κ B pathway was thought to elicit a pro-inflammatory response, but selective gene-deletion studies have shown that this pathway produces both beneficial and deleterious effects^[11]. NF κ B activation stimulates expression of numerous molecules that are involved in the inflammatory response such as IL-1 β , TNF, IL-6, IL-8, ICAM1 and other adhesion molecules^[11]. NF κ B also stimulates the expression of protective molecules, *CARD15*, cyclo-oxygenase 2, B defensins, TNF-induced protein 3 that inhibit the inflammatory response^[11].

IL-23 is a key cytokine in orchestrating the crosstalk between innate and adaptive immunity and has a central role in driving early responses to microbes^[35]. Interestingly, IL23R polymorphisms have been associated with both CD and UC, suggesting that the IL-23 axis might represent a shared inflammatory pathway in chronic intestinal inflammation^[35]. Recent studies have shown that, besides its activity on Th17 cells, IL-23 can also act on cells of the innate immune system. Unconventional, innate-like T cell populations, which are particularly represented at mucosal sites, have been found to respond to IL-23 stimulation and secrete Th17-related cytokines^[35].

The adaptive immune system is highly specific and confers long lasting immunity and is adaptable with its antigen specificity and maturation^[35]. The inhibitory cytokines IL-10 and TGF- β in Peyer's patches, mesenteric lymph nodes and lamina propria are involved in T-cell tolerance in the intestine^[10]. Immunologically Th0 cells are activated and differentiate into Th1, Th2 or Th17 cells based upon the clearance of specific pathogens, but a dysregulated T cell response with abnormal development of activated T cells subsets may lead to the onset of inflammation by an excessive release of cytokines and chemokines with have multiple pathogenic effects^[35]. Moreover, there is a genetic association between the inhibitory cytokine IL-10 and UC, in which IL-10 participates in the down-regulation of intestinal inflammation^[10]. CD had been thought to be a Th1 mediated disease, secreting copious amounts of IFN γ , TNF- α , and IL-12; whereas UC was thought to be a Th2 mediated response, with signature cytokine secretion from IL-4, IL-5 and IL-13^[15]. However, recent data has suggested this paradigm is not quite so straightforward^[15]. Furthermore, data suggests that Th17 cell production of IL-17 and IL-23 play important roles in the pathogenesis of IBD, with DCs isolated from CD patients producing more IL-23 than UC patients^[15].

Th17 cells are a subset of helper T-cells that are induced by IL-6 and TGF- β and expanded by IL-23^[15]. Signaling is mediated through the engagement of heterodimeric IL-23 with its heterodimeric receptor that activates the JAK-STAT signaling pathway, which then regulates the transcription of several genes^[10]. IL-23 secreted by macrophages and dendritic cells may contribute to Th17 proliferation, survival or both^[10].

PATHOPHYSIOLOGY OF DSS-INDUCED COLITIS: AN INVALUABLE ANIMAL MODEL

Numerous animal experiment in the last 25 years have employed the DSS colitis model as a chemical induction of intestinal inflammation model that morphologically and symptomatically resembles epithelial damage seen in human ulcerative colitis^[1,36,37]. The DSS model, initially reported by Okayasu *et al.*^[38], and has been used to investigate the role of leukocytes in the development of colitis in animal models and is now one of the most extensively employed experimental models abetted by its simplicity and reproducibility^[38,39].

DSS is a water soluble, negatively charged sulfated polysaccharide with a highly variable molecular weight ranging from 5 to 1400 kDa. Murine colitis results from administration of 40-50 kDa DSS added to drinking water. In the DSS model, the sulfated polysaccharide does not directly induce intestinal inflammation, but rather acts as a direct chemical toxin to colonic epithelium resulting in epithelial cell injury. The proposed and most accepted mechanism by which DSS induces intestinal inflammation results in the disruption of the intestinal epithelial monolayer lining, leading to the entry of luminal bacteria and associated antigens into the mucosa and allowing the dissemination of proinflammatory intestinal contents into underlying tissue^[2,4,6,38,40-42].

The effectiveness of the DSS-induced colitis depends on several factors, including DSS concentration (usually 1%-5%), duration and frequency of administration (acute or chronic), molecular weight of the manufactured DSS, strain of animals (C3H/HeJ, C57BL/6 and BALB/c mice strains are more susceptible), and microbial environment of animals (*i.e.* germ-free versus specific pathogen-free). Furthermore, depending upon these various factors, the animals may develop acute colitis, chronic colitis or even colitis induced dysplastic lesions (Table 1).

In various protocols, 40-50 kDa DSS is added to sterilized drinking water at various concentrations to produce the desired inflammatory effect. At this molecular weight the tissue distribution reveals that DSS penetrates the mucosal membrane of the intestine^[4]. Acute colitis is induced by administering DSS to the chosen strain of mice, often C57BL/6 or BALB/c male mice, for a period of 6-10 d (average 7 d). Alternatively, chronic colitis is induced by

Table 1 Factors that influence effectiveness of dextran sodium sulfate to induce colitis

Factors	Variables	Description
DSS	Molecular weight	40-50 kDa for tissue penetration (larger molecule does not penetrate colonic tissue well and smaller molecule has poor distribution)
	Dosage concentration	Ranges from 1.5%-3% used most frequently (1% with mild symptoms and delayed onset)
	Duration of therapy	Acute: 5-10 d administration
	Manufacturer/batch	Chronic: 4-5 repeating cycles of DSS and sterile water Various manufacturers with differing potency
Host	Genetically susceptible strain of animal	Certain strains are more susceptible to DSS colitis than other strains. Susceptible strains: C3H/HeJ, C57BL/6, BALB/C
Environment	Housing Conditions	Group <i>vs</i> individual unit, frequency of cage changes alters coprophagy by host
	Microbial State	Germ-free <i>vs</i> specific pathogen free <i>vs</i> wild type

DSS: Dextran sodium sulfate.

administering 4-5 repeated cycles of DSS, each cycle involves administration of various DSS concentrations for 1 wk followed by sterile water for 7-14 d^[1,38,43,44]. The severity of colitis can be augmented based upon the duration of DSS administration as well as the concentration of DSS^[1]. During DSS administration, mice can exhibit pronounced weight loss (about 5%-10% reduction by day 5), altered stool consistency leading to diarrhea and hematochezia^[41]. A significant physiological indicator of animal stress and imminent demise occurs if weight loss is greater than 20% of initial weight^[41]. Following the desired protocol, the mice are then sacrificed and tissue samples are collected for histological analysis and other assays^[8,41].

Observations of the chronological changes induced by DSS administration have revealed that the signs of disease appear as early as 1 d of treatment primarily identified by changes in the expression of tight junction proteins such as occludin, Zonula occludens-1 (ZO-1) and various claudins^[4,8,40]. The altered expression of tight junction complexes and increased epithelial apoptosis has been identified in human IBD and is thought to contribute to leaks in the epithelial barrier^[4]. These modest initial effects are followed by increasingly worsening symptoms, including increased intestinal permeability, severe bleeding and mortality^[8]. The typical histological changes induced by acute DSS include mucin and goblet cell depletion, epithelial erosion, ulceration and infiltration of granulocytes into the lamina propria and submucosa resulting in immune responses^[2,8]. In chronic DSS protocols, additional histological changes such as crypt architecture disarray and widening of the gap between the base of the crypt and muscularis with deep mucosal lymphocytosis tend to appear a few weeks after DSS induction^[8]. Furthermore, transepithelial migration of neutrophils resulting in cryptitis and crypt abscess, a common histologic finding in human IBD, is reproduced in mice subjected to the chronic DSS administration^[8]. With prolonged administration of DSS in rodents, squamous metaplasia of rectal mucosa, adenomatous changes and adenocarcinoma can also be seen histologically^[45].

It has been identified that the molecular weight of DSS is critically important to the induction, severity

and of DSS induced colitis^[4]. Carcinogenic activity in the colon is achieved by DSS of 50 kDa while larger and smaller molecular weights (520 kDa and 10 kDa, respectively) failed to induce activity due to inadequate tissue penetration^[4]. Additionally, the repeated pulses of DSS administration utilized in chronic phases of DSS colitis results in dysplasia that frequently resembles the clinical course in human UC^[31].

The knowledge of the unique or exact mechanisms that result in carcinogenesis underlying colitis-associated cancer (CAC) in humans is lacking. The transition from inflammation, to dysplasia and hence cancer is not fully elucidated, but it has been proposed that a host of multiple factors are integral in the role of CAC including; immune response, activation of oncogenes, inhibition of tumor suppressors, as well as alterations in intestinal microbiota^[46]. A variety of murine models of CAC have been developed. The most widely used and best studied is a chemically induced colitis-associated model that incorporates a combination of a potent carcinogen, azoxymethane (AOM) and DSS^[47]. Long term administration or repeated cycles of DSS has been shown to induced chronic colitis and subsequent dysplasia in rodents^[48]. In this murine model, as in human CAC, the degree of inflammation correlates with dysplasia and is associated with nuclear translocation and mutational activation of β -catenin which results in increased activity of the Wnt signaling pathway^[46,48]. The change in Wnt signaling results in enhanced inflammatory immune response with release of pro-inflammatory cytokines (IL-6 and TNF- α), which results in elevated levels of c-myc, a known oncogene and activator of cell cycle progression^[46]. Additionally, many important inflammatory components are increased and activated during CAC (NF κ B, Janus Kinase, cyclooxygenase-2 and inducible nitric oxide synthase) leading to further infiltration of lymphocytes, plasma cells and macrophage to sites of chronic inflammation^[46]. The limitation of the models is that Kras or p53 mutations, that are typical in human CAC, are not present in murine AOM/DSS-induced CAC models^[46].

DSS colitis is associated with increased production of various cytokines and chemokines. Following

the induction of DSS colitis various tissue derived cytokines have been shown to be upregulated as early as the first day of DSS-induction^[4]. The different inflammatory mediators assessed include TNF- α , the hallmark of DSS induced colitis, IL-6, IL-10, IL-17, IL-1 β , TGF- β , mucin, TLR2/4 gene expression, MPO activity^[1,4]. Differences in inflammatory profiles was expressed between acute and chronic DSS phases^[4]. It was shown that in acute inflammation in DSS converts to a predominant Th-2 mediated response in the chronic state with noted decreased levels of TNF- α , IL-17 and elevated levels of IL-4,-6,-10 and IFN γ ^[4,49]. IL-6, IL-1 β tissue levels correlate with IBD activity and IFN γ secretion has been linked to IL-17 secretion which tends to be expressed during chronic inflammation^[31]. It has been shown that the cytokine profiles in DSS colitis phases correlates with barrier function, histological and clinical parameters lending the model as an integral tool in the study of cytokine role in induction and recovery from inflammation^[4]. The bulk of research on IBD pathophysiology till date has focused on the immunological response and less attention has been paid to defects in barrier function that can potentially increase bacterial contact with the epithelium^[50]. In animal studies assessing epithelial damage, DSS has been considered a toxicity model as induction of intestinal inflammation is not direct. Rather the chemical exerts an epithelial injury resulting in intestinal epithelial barrier disruption causing an influx of luminal bacteria and associated antigens into the mucosa and submucosa and thus triggering an inflammatory reaction^[51]. An alternative mechanistic action for the induction of inflammation in the DSS model is through dysbiosis of murine gut microbiota leading to immunoregulation defects, mucin and goblet cell depletion and barrier dysfunction. Although the exact mechanism through which DSS induces colonic mucosal inflammation is not completely understood, recent results indicate that sulfate groups of the DSS molecules destabilize the mucus layers and make it more permeable to bacteria^[52]. Hence, the DSS model is not simply a toxicity model, but also a barrier dysfunction model that encompasses mucus loss and the eventual bacterial penetration frequently found during intestinal trauma^[53].

DSS DISRUPTS INTESTINAL BARRIER FUNCTION

The function of the intestinal epithelium is to simultaneously provide a barrier between the host and external environment while facilitating selective permeability that limits migration of harmful molecules but allows appropriate absorption of nutrients, ions and water^[19]. This dynamic relationship of selective permeability is dependent upon specialized structures composed of tight junction complexes^[40]. Tight junctions (TJ) are protein complexes consisting of

Occludin, ZO, Claudins and junctional adhesion molecules (JAM) that are located at the apical ends of the lateral membranes of IEC and form a physiologically active barrier that can alter permeability based on the cellular environment^[40,54-56].

Dysfunctions of intestinal barrier lead to increased intestinal permeability that have been associated with the pathogenesis of IBD^[2]. Barrier dysfunction in IBD has been identified as shifts in tight junction protein expression, and function with poorly adherent mucosa in the inflamed intestinal mucosa^[2,40,43,54,57,58]. This change in composition and function corresponds to an increase in intestinal permeability with entry of commensal bacteria and decrease in transepithelial resistance^[2,40,57]. The question remains whether the alteration in the intestinal barrier function is a primary necessity for the inflammatory response or a secondary development from the inflammatory response^[40].

Several studies have shown that the appearance of intestinal inflammation is not the initial, inciting event, but rather the TJ complex changes and the subsequent increase in colonic permeability precede the development of intestinal inflammation^[38,40,42,43,57,59]. In mice DSS colitis leads to a decrease in TJs expression that is followed by an increase in permeability and clinical manifestations of colonic inflammation^[19]. Specifically, the protein pattern of TJ undergo rapid changes, as evident in the increased expression of claudin-2 and depletion of various claudins and zona occludens-1^[19,40,54]. Therefore, the breach in the mucosa barrier is seen as a secondary event to the increase in colonic mucosal permeability resulting in the influx of inflammatory cells into the intestinal mucosa^[40,57].

As noted earlier, the TJ are comprised of protein complexes that include occludin, ZO, claudins and junctional adhesion molecules. Importantly, claudin proteins are intrinsically involved in the formation of the IEC barrier function^[60]. Claudins comprise a multigene family of 27 members and are expressed in a pattern that is both organ and segment-specific. A majority of these claudins (claudins 1, 3, 4, 5, 7, 8) confer barrier properties and are often found in tight epithelia of distal intestine. Claudins interact in a tissue-specific manner to form a charge-selective and size-selective barrier and predominantly contribute to epithelial barrier function and regulate paracellular permeability in intact epithelium^[61]. Several mouse studies have assessed the expression of various claudins in conjunction to DSS administration. So far, the results have been varied. One study found enhanced expression of the claudin-1 protein, whereas other studies found decreased expression of claudin-1^[54,60]. These contradictory findings could be related to species differences as claudin-1 was increased in rats while decreased in mice when exposed to DSS. Further studies conducted by independent laboratories reported similar findings demonstrating an up-regulation of the pore-forming claudin-2 and decreased expression

in claudin-3,-5,-7,-8^[60]. Furthermore, alterations in claudin expression resulted in similar outcomes with pronounced barrier dysfunction with aggravated mucosal damage and increased colonic permeability in DSS-induced colitis^[60].

Claudin-2 has been shown to provide a critical role in regulating colonic epithelial homeostasis and barrier function; therefore, regulating mucosal immune response and mucosal inflammation. A frequent regulatory step in inflammation is the increased expression of claudin-2 and its insertion into TJ strands. Upregulation of claudin-2 expression is found to start in the lower crypt and progress toward the surface epithelium^[56]. The functional role of this modified expression and the consequential increase in intestinal permeability is still uncertain^[56]. Consequently, recent human studies have examined potential alterations in claudin family functions in IBD patients and demonstrated a robust increase in claudin-2 expression^[62]. Similarly, claudin-2 was found to assist the uptake of mucosal antigens^[62]. A corollary finding in DSS-treated CI-2TG mice revealed significant suppression of pro-inflammatory molecules with overexpression of claudin-2 suggesting its pivotal role in immune adaptation^[62]. The paradoxical finding of increased regulatory CD4+ cells of unchallenged mice and decreases in immune cell infiltration of DSS-challenged mice suggests that claudin-2 induced epithelial permeability facilitates the interaction of host immune molecules and luminal antigens to promote adaptive tolerance and protection from colitis rather than increased sensitization^[62]. Additionally, the DSS-challenged mice showed decreased apoptosis and increased epithelial proliferation further supporting the role of claudin-2 in intestinal epithelial cell regulation^[62]. Conversely, claudin-2 knockout mice subjected to DSS exhibited severe colitis^[62].

In addition to changes seen in claudin expression, other studies assessing barrier dysfunction assessed changes in ZO-1. Merely one day of DSS administration caused a statistically significant reduction of ZO-1^[40]. Thus, the loss of TJ integrity led to an increase in permeability which occurred before any significant clinical or histological evidence of colitis^[40]. Another study supporting this premise reported a redistribution of occludin and ZO-1, from the junctions in colonic epithelium after 4 d of administration of DSS^[42]. As seen in the claudin studies, the ZO-1 data presented suggests that TJ complex changes are a prerequisite for the development of intestinal inflammation^[40,42].

Another adhesion molecule with essential roles in the development and homeostasis of several tissues is the large group of proteins E-cadherin, that represent the major component of adherens junctions^[63]. Studies suggest that E-cadherin may factor into the pathogenesis of UC as mice with E-cadherin deficiency had more severe colitis in the DSS model^[63]. Further, similar to redistribution of occludin, ZO-1, E-cadherin and B-catenin also translocate from the junctions in

colonic epithelium after 4 d of DSS treatment^[2,42].

DSS EFFECTS ON MUCIN

Observations in several human studies found patients with colonic inflammation had alterations in colonic mucus and decreased effectiveness in its barrier function^[64]. The gastrointestinal tract is quite remarkable with respect to the protective mucus barrier organization. The secretory, gel-forming mucins form the outer loose layer of mucus and the inner dense membrane-bound mucins covers and protects the surface epithelial cells^[64,65]. Biochemically, mucins are usually very large, filamentous molecules with a large region within their polypeptides, which comprise relatively short tandemly repeated peptide domains which are highly O-glycosylated^[66]. In humans, fifteen different mucins have been described and are assembled in the MUC gene family, with only a few encoded for activity in the colon^[64,66]. Of the mucins, only Muc2 has been shown to be the principle secretory gel-forming mucin in both large and small intestines providing the functional barrier between epithelium and microbiota^[64,65]. Muc2 is the predominant mucin produced by intestinal goblet cells, and is thickest in the healthy colon^[67]. Whereas, the other membrane bound mucins with transmembrane regions are involved in cell signaling, adhesion, growth and modulation of the immune system^[64,66].

As mucus is in direct contact with many microorganisms within the intestinal tract, defects in gene coding or protein folding of the mucins could lead to poor membrane integrity and ultimately a breach in the epithelial barrier or alterations in the mucosal-bacterial interactions. Prior *in-vitro* studies on intestinal cell lines revealed that the mucin expression and structure is influenced by cytokines, bacteria and their associated components^[66,67]. Likewise, in UC the alteration of immunological or bacterial factors can influence mucin production^[67]. Goblet cell depletion is a frequent histopathology finding in UC patients implying that the synthesis of Muc2 is decreased in association with smaller goblet cells thecae and histologic appearance of goblet cell depletion^[67]. This, in turn, leads to further adverse effects resulting in chronic inflammation that is characteristic of IBD^[64,66]. With these considerations in mind, it is still uncertain whether mucin decrease and goblet cell depletion are the primary contribution to IBD or the consequences of inflammation^[67].

Mice deficient in Muc2 are characterized by a loss of mucus layer with bacteria not only in direct contact with epithelial cells but found deeper in the crypts (Table 2). These observations are absent in healthy animals. Furthermore, mice deficient in Muc2 ultimately are more susceptible to spontaneous severe colitis and eventually cause an increased risk of colon cancer development^[52,68-70]. Beyond DSS mouse models, other models with defective mucus

Table 2 Role of the different classes of mucins in dextran sodium sulfate induced colitis

Mucin gene	Mucin class	Expression site (GI tract)	Chromosome	Pathological findings with DSS treatment	Ref.
<i>Muc1</i>	Membrane bound	All epithelia	3	<i>Muc1</i> ^{-/-} protective when challenged with DSS Increased thickness with adherent mucus	[64,66]
<i>Muc3</i>	Membrane bound	Intestine, enterocytes	5	Increased up-regulation of <i>Muc3</i> gene	[65,66]
<i>Muc4</i>	Membrane bound	All epithelia	n.d.	Large type 1 transmembrane glycoprotein <i>Muc4</i> ^{-/-} more resistant to colitis due to up-regulation of <i>Muc2</i> due to increase proliferation of cytokines	[65,66,73]
<i>Muc2</i>	Secretory	Intestine, goblet cells	7	<i>Muc2</i> ^{-/-} more susceptible to spontaneous colitis, increased risk of CAC DSS produces fulminant colitis <i>vs</i> wild type	[66-68]
<i>Muc5ac</i>	Secretory	Stomach	7		[66]
<i>Muc5b</i>	Secretory	Tongue, sublingual glands	7		[66]

CAC: Colitis-associated cancer; DSS: Dextran sodium sulfate.

function all develop colitis^[50,71,72]. In order to fully comprehend the role of mucins, specifically *Muc2*, in epithelial barrier protection and colitis, studies were conducted on *Muc2* deficient mice. DSS administration to these mice produced fulminant colitis within days compared to the treated wild-type mice^[68]. Further, a single missense mutation in *Muc2* lead to spontaneous colitis with increased intestinal permeability and increased cytokine production in the distal colon^[67]. Additionally, during DSS induction the thickness of the mucus gel decreased at the same time as the mice developed increasing colitis symptoms^[64]. Analysis of *IL-10*^{-/-} mice revealed thicker mucus layers than wild type, but in further analysis specific mucin properties were altered and the usually impenetrable inner layer was found to be penetrable to intestinal bacteria^[52]. Therefore, mucus thickness alone was not shown to be a meaningful predictor or indicator of mucus barrier function^[52].

Post-translations modifications on mucin are key to their functionality. Along these lines, an increased levels of *Muc2* seen following DSS administration fails to control inflammation because of decreased sulphation in *Muc2*^[66,67]. Further studies have shown that alteration in glycosylation of mucins resulted in decrease *Muc2* synthesis and a diminished mucus barrier, thereby increasing the susceptibility to DSS-induced colitis^[67]. The *IL-10*^{-/-} germ free mice given normal enteric bacteria, presenting with a loss of sulphation of newly synthesized *Muc2* molecules, were more prone to develop colitis and produced severe and chronic colitis^[66]. Collectively, these observations underscore the importance of post-translational modification on mucin in its functional role in regulating intestinal barrier integrity^[67].

In conjunction with the secretory mucins, the various cell surface mucins are considered to serve a critical function in mucosal protection^[65,67]. *Muc4* is a large type- I transmembrane glycoprotein component normally expressed on the surface of colonic epithelial cells. *Muc4*^{-/-} mice were more resistant to colitis and CAC induced by AOM/DSS because of the compensatory upregulation of *Muc2* expression in

these mice^[65]. Although not statistically significant, an upregulation of *Muc3* (orthologue of human *Muc17*) was also observed^[65]. Additionally, *Muc4*^{-/-} mice induced with DSS had increased expression of pro-inflammatory cytokines (TNF- α and IL-1 β) which could also be responsible for the observed upregulation of *Muc2* and *Muc3*^[65]. Although both *Muc4* and *Muc13* belong to the transmembrane mucin subtype, deletion of either of the two mucin genes results in opposing phenotypic response to DSS treatment. *Muc13*^{-/-} mice had increased macrophage expression in the inflamed mucosa accompanied by increased expression of intestinal IL-1 β and TNF- α mRNA and significantly increased loss of body weight, diarrhea score, fecal blood score and severe histologic damage including intestinal epithelial cell apoptosis following DSS exposure. This suggests *Muc13* confers protection to colonic epithelial cells from apoptotic stimuli, preventing damage-induced cell death^[67]. In contrast, *Muc4*^{-/-} mice exhibit a protective phenotype (decreased loss of body weight, diarrhea score, fecal blood score and less severe histologic damage) which is related to a compensatory upregulation of *Muc2* and *Muc3*^[65,73].

Muc1, a non-gel forming mucin transmembrane mucin, when deleted resulted in protection of colonic epithelial cells similar to findings in *Muc4*^{-/-} mice when challenged with DSS^[64]. *Muc1*^{-/-} deficient mice had increased thickness of adherent mucus resulting in mild colitis^[64]. Additionally, germ-free mice had a thin colonic mucus barrier, but luminal exposure to the bacterial products lipopolysaccharides (LPS) and peptidoglycan quickly restores the firmly adherent mucus layer thickness to levels observed in conventionally housed mice^[64]. *Muc1*^{-/-} mice developed a more severe inflammatory response after exposure to *H. pylori* compared with wild-type mice, demonstrating that cell surface mucins can modulate the inflammatory response to chronic infection^[67]. As opposed to observations in *Muc2* deficient mice resulting in severe colitis, the *Muc1*^{-/-} mice developed a very mild colitis which is thought to be due to an increased colonic mucus barrier and a decreased ability to recruit T cells to the affected region^[64].

DSS ALTERS THE MICROBIAL BALANCE

The intestinal microbiome plays an integral role in host immune development, tolerance and intestinal physiological processes. This has spurred large collaborative efforts aimed at identifying and characterizing the microorganisms which are associated with the health and disease in humans^[25,32,74,75]. However, the sheer presence of microbiota in the intestinal tract alone is not enough to exert these physiological effects, rather variant microbe compositions, temporal changes in populations and relative abundance of specific microbes are important in homeostasis and specific disease states, namely IBD^[66,74-76].

Effects on mucosal inflammation

Studies involving DSS-induced colitis have revealed the critical role that gut microflora play in the pathogenesis of mucosal inflammation and the related role of barrier function as a bulwark against extensive stimulation of the mucosal immune system. However, the exact mechanism through which bacteria induce inflammation has been elusive^[22,76]. Indeed, a number of recent studies have identified compositional changes at the bacterial phyla and species levels that can influence phenotypic expression of both pro- and anti-inflammatory responses in humans and in murine models relevant to IBD^[22,25,74,76].

As indicated earlier, microbiome composition alterations can result in normally underrepresented members of the microbiome to become dominant, leading to perturbations in structure and function of the microbiome. Various DSS-induced colitis studies have noted that murine microbiota alterations occurred early (within 3 d) and are characterized by diversity reduction and changes occurred prior to the clinical or biochemical evidence of inflammation^[31,75,77]. Composition changes in DSS-induced murine colitis indicated a significant proliferation in Bacteroidaceae and Clostridiaceae families^[38]. Further studies have corroborated these initial findings to suggest dramatic reductions in the genera of Bacteroidetes, Prevotella, Clostridium and Lactobacillus with corresponding increases in pro-inflammatory gut microbiotic components Bacillaceae, Enterococcales and Enterobacteriaceae^[31,78,79].

Effects on barrier functions

Beyond the simple changes in microbiome composition that may herald alterations in physiologic homeostasis, other recent studies have examined the role that bacteria influence barrier function alteration. Evidence suggests that epithelial integrity is compromised in DSS-induced colitis leading to penetrance of microbes and associated antigens into the mucosa and^[69,75,80,81]. One such study examining the anti-inflammatory effect of *Faecalibacterium prausnitzii* suggested that

its immunomodulatory effects are mediated *via* decreasing the paracellular permeability to effectively reduce the severity of colitis and prevent colitis progression. *Faecalibacterium prausnitzii* decrease NF- κ B activation and IL-8 secretion *in vitro* and impairs the colonic synthesis of pro-inflammatory cytokines while inducing the secretion of anti-inflammatory cytokine *in vivo*^[19]. Likewise, various strains of bifidobacteria have shown promising anti-inflammatory effects^[82]. Specifically, strains of *Bifidobacterium bifidum* have been reported to inhibit LPS-dependent NF- κ B activation in IECs and induce anti-inflammatory macrophage, dendritic, and T cell populations^[82].

Effects on mucin

Metagenomics studies following the administration of DSS revealed an elevation in *Akkermansia muciniphila*, a Verrucomicrobia member, which have been found to metabolize sulfur and lead to mucin degradation and correlate with disease activity in mice administered DSS^[31,75,78]. Additionally, DSS induced colitis obliterated the difference in abundance and structure of bacterial communities between the two mucus layers^[52,69]. There were fewer bacterial in the firmly adherent mucus but the abundance of bacteria in the mucus layer of the DSS treated animals was 10-100 times greater compared to the control group, suggesting DSS treatment increased the total count of infectious bacteria^[22]. Antibiotic administration improved DSS-induced colitis akin to observations showing reduced inflammation in germ-free murine colitis model^[31,32].

Changes during the recovery phase

Interestingly, there is a rapid shift of the gut microbial community toward a healthy profile in the recovery phase following the DSS treatment regimen. Within two days of stopping DSS administration, the mice gut microbiota showed relative abundance in Bacteroidetes/Prevotella, Bacillaceae, to levels comparable to those observed in healthy controls^[31]. These data demonstrated the high degree of resilience of the gut microbiota and a rapid recovery of its healthy mutualistic profile after DSS-induced dysbiosis^[31].

Probiotic treatment against dextran sodium sulfate-induced colitis

Probiotic therapies with Lactobacilli and Bifidobacteria produced favorable outcomes in murine colitis models^[82,83]. Probiotic bacteria have been shown to decrease intestinal permeability and restore gut barrier integrity by modulation of tight junction proteins^[83]. Pretreatment with *Lactobacillus reuteri* strains prevented the onset of DSS-induced colitis by reducing bacterial translocation and suppressing adherence of lactobacilli on colonic mucosa and^[22]. Further, probiotic treatment suppressed upregulation of P-selectin in the colonic endothelium, which decreased leukocyte-

endothelial cell interactions and concomitant leukocytes recruitment to tissue^[22]. However, no significant changes in microbial composition were observed indicating that the protective effects are linked to strengthening of the epithelial barrier integrity to reduce bacterial translocation^[22,69]. Other studies have also examined the effects of different bacterial species on epithelial TJ in the DSS-induced colitis model. Various strains of bifidobacteria have shown promising anti-inflammatory effects^[83]. *Bifidobacterium bifidum* S17 has been found to adhere to cultured IEC and displays potent anti-inflammatory activity both *in vitro* and in two murine models of colitis^[83]. *Bacillus subtilis* supplementation resulted in improved barrier function compared to the DSS group, as evident by upregulated expression of TJ proteins (claudin-1, occludin, JAM-A, and ZO-1) and downregulated cytokine expression (IL-6, IL-17, IL-23, and TNF- α)^[84]. Additionally, only certain, limited strains of *Bifidobacterium longum* have indicated an increased expression of ZO-1 and occludin resulting in reduced severity of DSS-induced colitis^[83].

DEXTRAN SODIUM SULFATE-INDUCED CHANGES IN METABOLOME

DSS colitis has been shown to produce disturbances in the metabolism of phospholipids depicted by decreased levels of phosphocholine and glycerophosphocholine in the colon of mice^[1]. Phosphocholine and glycerophosphocholine are the most important metabolites of choline and the major cellular constituents required for the assembly of biological membranes and disturbance in the metabolism suggests the possibility of distorted membrane integrity in the presence of DSS^[1].

The role of dietary fat intake in exacerbating intestinal inflammation and modulation of immune function has been investigated extensively^[85]. There is supporting evidence to show that a high fat intake is associated with an increased risk of ulcerative colitis^[85]. Of note, two factors determine the role of lipid nutrition in health and disease: (1) the composition and (2) the total amount of fat in the diet. Bile acids are produced in the liver and excreted into the duodenum as conjugated bile salts to facilitate homeostatic functions in the gastrointestinal tract. Once in the large intestine, various microbial species enable the transformation of the bile salts into secondary bile acids, such as deoxycholic acid and urosodeoxycholic acid (UDCA), which vary in hydrophobicity^[86]. Increased luminal bile acid hydrophobicity reportedly leads to gut barrier dysfunction through the disruption of cell membranes causing cytotoxicity, production of reactive oxygen species, epithelial growth factor receptor activation and tight-junction redistribution^[86]. Conversely, UDCA which is more hydrophilic leads to stabilization of lipid membranes^[86]. Thus, changes in bile acid composition favoring increased hydrophobicity leads to increased

gut permeability allowing for enhanced translocation of bacteria and associated antigens across the tight-junction barrier of the gut epithelium leading to intestinal inflammation. One such study identified animals with a lower concentration of fecal cholic acid had increased histologic damage^[86,87]. Additionally, various types of dietary fats promote colitis *via* alterations in gallbladder bile and gut microbiota through changes in taurocholic acid levels in bile which facilitated the growth of the *Bilophila wadsworthia*, an inflammatory Gram-negative anaerobe^[86,88]. Overall, fecal bile acid hydrophobicity found to be mediated through a higher proportion of deoxycholic acid positively correlates with the severity of DSS colitis^[86].

A potential causal link between dietary fat, especially Omega-3 and Omega-6 fatty acids, and susceptibility to induced colitis has also been reported. A dose dependent effect of the changes in composition of omega fatty acids in exacerbating colitis in various models including the DSS colitis model has been reported^[85,86]. Fat in the diet alone does not alter the susceptibility to DSS, rather the modifications to the fecal bile acid composition has been proven to be deleterious^[85]. Omega-3 fatty causes decreased adiponectin mRNA expression acid leading to increased inflammation in colonic mucosa^[85]. However, another study reported decreases adiponectin RNA expression with no changes in serum adiponectin levels by high fat diet^[85]. There are also reports that suggest that the pro-inflammatory effect of adiponectin is mediated by increased IL-6 production^[85].

In addition to studies examining primary effect of bile acids on inflammation, the effects that bile acids on cell surfaces have also been investigated^[89]. A member of the G protein coupled receptor superfamily, GP-BAR1 is a cell surface bile acid-activated receptor that has been found to be highly expressed in the ileum and colon and activated by secondary bile acids, specifically lithocholic acid and tauro-LCA^[89]. Cipriani *et al.*^[89] asserted that this receptor regulates intestinal barrier integrity since mice lacking the GP-BAR1 receptor developed alterations in colonic histopathology and mucous cell distribution and function^[89]. GP-BAR1^{-/-} mice challenged with DSS exhibited an exacerbation of colitis that was not correlated with major immunological abnormalities, but rather an increase in intestinal permeability^[89]. Furthermore, they have discovered that oleanolic acid, a natural GP-BAR1 ligand, attenuated colon inflammation and these anti-inflammatory effects of ciprofloxacin were lost in GP-BAR1^{-/-} mice^[89].

The central argument that metabolome changes play a part in colitis development is highlighted by the critical role microbiome composition plays in bacterial-host immune response. Certain enzymes, such as 7 α -dehydroxylase that are endemic to gut microbiota, can convert primary bile acids into their secondary forms and therefore result in pro-inflammatory versus anti-inflammatory conditions in the host gut lumen.

Additionally, shifts in the community composition and structure can reduce potentially immunomodulatory mucosal-associated species, such as *Faecalibacterium prausnitzii*, *Clostridium leptum* and *Clostridium coccoides*, that act to maintain epithelial health through the production of short-chain fatty acids and stimulation of mucin production, thereby effecting the host's inflammatory response^[25,27,75,90].

CONCLUSION

The last several years have provided indispensable insights into the histopathological and morphological changes in intestinal barrier function that likely contribute to the development and progression of murine colitis and aids in the understanding to the pathogenesis of human IBD. As stated prior, no isolated model has proven to sufficiently represent the complex clinical and histopathological characteristics of human disease. But, the DSS-induced colitis model is the most commonly used model that provides an inexpensive, simple and reproducible model to study various aspects of the role of mucin in barrier integrity, alterations in microbial balance and changes in the metabolome that relate to the pathogenesis of IBD. It is our hope that our further understanding of the role of intestinal barrier function in activating the innate and adaptive immune system in the intestinal tract that may lead to new therapeutic targets for human IBD.

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Autoimmune hepatitis: Standard treatment and systematic review of alternative treatments

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Abstract

Autoimmune hepatitis is a rare chronic inflammatory liver disease, affecting all ages, characterised by elevated transaminase and immunoglobulin G levels, positive autoantibodies, interface hepatitis at liver histology and good response to immunosuppressive treatment. If untreated, it has a poor prognosis. The aim of this review is to summarize the evidence for standard treatment and to provide a systematic review on alternative treatments for adults and children. Standard treatment is based on steroids and azathioprine, and leads to disease remission in 80%-90% of patients. Alternative first line treatment has been attempted with budesonide or cyclosporine, but their superiority compared to standard treatment remains to be demonstrated. Second-line treatments are needed for patients not responding or intolerant to standard treatment. No randomized controlled trials have been performed for second-line options. Mycophenolate mofetil is the most widely used second-line drug, and has good efficacy particularly for patients intolerant to azathioprine, but has the major disadvantage of being teratogenic. Only few and heterogeneous data on cyclosporine, tacrolimus, everolimus and sirolimus are available. More recently, experience with the anti-tumour necrosis factor- α infliximab and the anti-CD20 rituximab has been published, with ambivalent results; these agents may have severe side-effects and their use should be restricted to specialized centres. Clinical trials with new therapeutic options are ongoing.

Key words: Autoimmune hepatitis; Standard treatment; Second-line treatment; Adults; Children

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Core tip: The first part of this review summarizes the standard therapeutic approach for autoimmune hepatitis (steroids and azathioprine) and the evidence on which it is based. The second part reviews systematically published data on first and second line alternative treatments. This information is summarized in two comprehensive tables, one for adult and one for paediatric patients.

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INTRODUCTION

Autoimmune hepatitis (AIH) is a rare inflammatory liver disease of unknown origin characterised by high transaminase and immunoglobulin G (IgG) levels, positive autoantibodies, and, histologically, by interface hepatitis^[1-4]. The condition affects all ages, and has a female preponderance^[5]. There is no single diagnostic test^[1,2]. The International Autoimmune Hepatitis Group (IAIHG) established comprehensive diagnostic criteria in 1993^[6], based on expert opinion, intended to be used for research purposes. After their evaluation in a number of studies, the criteria were updated in 1999^[7]. A simplified, clinical practice-friendly version was published in 2008^[8]. These criteria are intended to help in guiding diagnosis and decision on therapy initiation in patients presenting with a clinical picture suggesting AIH, and have received extensive external validation since publication^[9-11].

AIH is divided in type 1 and type 2, the latter being rare in adults and representing 30% of juvenile AIH. The distinction is made serologically: type 1 AIH is positive for anti-nuclear antibodies (ANA), and/or anti-smooth muscle antibodies (SMA), while type 2 AIH is positive for anti-liver kidney microsomal antibodies type 1 (anti-LKM1) and/or anti-liver cytosol type 1 (anti-LC1)^[12].

AIH is the first liver disease for which pharmacologic treatment has been shown to improve survival. Indeed, it has an excellent response to steroid-based immunosuppressive therapy, with a reported response rate of 75%-90%^[2]. Steroid-response is a crucial feature of AIH, and it is part of the IAIHG revised diagnostic criteria^[7]. Lack of response to steroids should prompt a review of the diagnosis.

Treatment indications

If untreated, AIH has a severe prognosis. This knowledge derives from early clinical trials, when "HBsAg-negative hepatitis" (as AIH was called then)

patients were treated with corticosteroids vs placebo. One placebo controlled study reported a 5-year survival rate of 32% in untreated patients vs 82% in patients treated with steroids^[13]. According to the guidelines on the management of AIH by the American Association for the Study of Liver Diseases (AASLD)^[2], the 6-mo survival rate in untreated patients is about 60%. Therefore, once diagnosed, AIH should be treated promptly. Elderly patients with mild paucior a-symptomatic disease, who have a high risk of developing steroid side effects, may be an exception, and in this clinical context treatment vs watchful waiting should be carefully evaluated case by case^[14-16]. Untreated patients need a close follow-up. Treatment must be always initiated in the presence of clinical symptoms, severe biochemical and/or histological disease activity. Younger subjects, particularly children and adolescents, who have a more aggressive disease, should be treated without delay^[17].

Treatment aims

The aim of treatment is disease remission, which is reached if the following criteria are met: (1) absence of clinical symptoms; (2) normal transaminase levels; and (3) normal IgG levels. In children/adolescents, negative or very low-titre autoantibodies (< 1:20 for ANA/SMA; < 1:10 for anti-LKM1) are an additional criterion of remission^[3], which remains to be evaluated in adults by longitudinal studies.

In the past, transaminase levels below twice the upper limit of normal (ULN) have been considered proof of remission, but it is now clear that patients with abnormal transaminase levels have progressive disease^[2,18]. Once remission is achieved, the lowest possible dose of immunosuppressive drugs should be used to maintain long-term remission with no or minimal side effects.

Disease relapse is defined as transaminase levels rising above the ULN after remission^[12]. Relapse occurs mostly if the dose of the immunosuppressive drugs is reduced, or in case of non-adherence. Non-adherence is a frequent clinical problem, particularly in adolescents^[19] and young adults, and is often due to real or perceived treatment side effects. It should always be suspected in case of relapse while on a stable dose of immunosuppressive drugs.

AIM AND METHODOLOGY OF THE SYSTEMATIC REVIEW

The aim of this review is, in its first part, to critically summarize the evidence on which standard AIH treatment (prednisone and azathioprine) is based, and, in its second part, to provide a systematic review of the published data on alternative treatments. For the purpose of the systematic review of the literature on alternative AIH treatment, publications cited in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) were

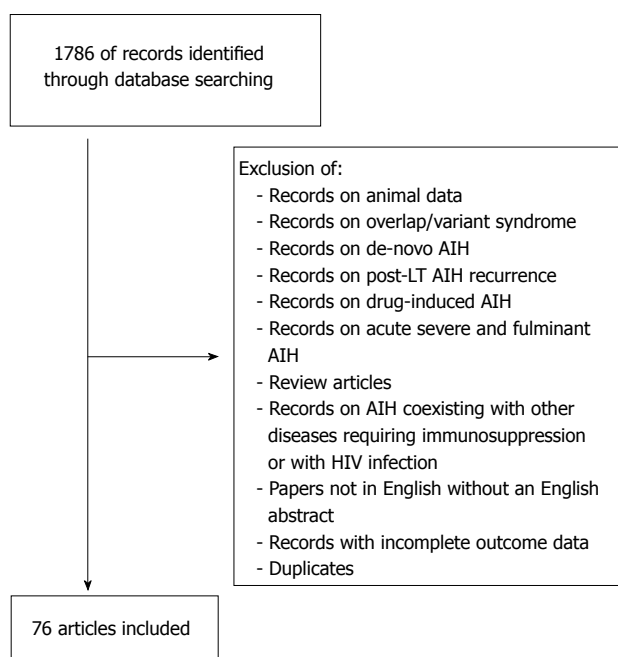


Figure 1 Selection of relevant articles for the systematic literature review on alternative AIH treatments. AIH: Autoimmune hepatitis; LT: Liver transplantation.

Table 1 Proposed schedule of prednisone tapering during remission-induction therapy in adults^[25]

	Prednisone mg/d	Azathioprine
Week 1	60.0	Check
Week 2	50.0	transaminase
Week 3	40.0	levels every
Week 4	30.0	week before
Week 5	25.0	reducing the
Week 6	20.0	prednisone dose:
Week 7	15.0	if transaminase
Week 8-9	12.5	levels stop
Week 10-11	10.0	decreasing, add
If severe steroid side effects:		azathioprine 1-2
consider reducing to 2.5 mg/d		mg/kg per day,
for 2 wk and then stopping		if jaundice is
prednisone		subsiding

Table 2 Proposed schedule of prednisone tapering during remission-induction therapy in children^[2,12]

	Prednisone mg/kg/d	Azathioprine
Week 1	2.0	Check transaminase
Week 2	1.75	levels every week
Week 3	1.50	before reducing the
Week 4	1.25	prednisone dose: if
Week 5	1.00	transaminase levels
Week 6	0.75	stop decreasing, add
Week 7	0.50	azathioprine starting
Week 8-9	0.25	with 0.5 mg/kg
Week 10-11	0.10-0.20	per day, if jaundice
If severe steroid side		is subsiding, at
effects: consider reducing		increasing doses up to
to 2.5 mg/d for 2 wk		2-2.5 mg/kg/d until
and then stopping		biochemical control
prednisone		

selected using the search words “autoimmune hepatitis” and “treatment”. Citations were chosen on the basis of their relevance to the aim of this article (Figure 1). Fundamental characteristics of the abstracts judged pertinent to the review were noted, and full-length original articles were selected from the abstracts. Seventy-six articles were identified, 22 of them are not discussed in this review because of anecdotal reporting, the remaining 54 are included in Table 1 (adults) and Table 2 (children). Children/adolescents have a more aggressive disease, with a more frequent acute presentation^[20] and therefore need a different management^[17]. For this reason, the present review article discusses adult and pediatric treatment separately.

STANDARD TREATMENT

Why do we treat autoimmune hepatitis with steroids and azathioprine?

Standard treatment is based on steroids and azathioprine (Table 1). A systematic review of randomized controlled trials focused on these two drugs up to 2009 was published in 2010^[21]. The exact azathioprine mechanism of action is unclear, but it is most probably linked to suppression of nucleic acid synthesis. The first evidence for steroid benefit in inducing remission and improving survival in treatment-naïve AIH stems from three trials performed in the 1970s, which demonstrated a significant better survival in patients with so called “HBsAg-negative chronic active liver disease” treated with steroids^[22-24] in comparison to untreated patients. It should be noted that at that time the hepatitis C virus (HCV) had not been discovered and it is likely that some patients with HCV were included in the trials, although “HBsAg-negative chronic active hepatitis” was characterised by high globulin levels, female preponderance, and presence of autoantibodies, all features of AIH^[13]. The benefit of steroid treatment would have probably been even greater if HCV-patients had been excluded^[25]. In the Royal Free Hospital trial^[22], 49 well characterised patients, including children, were randomised in a steroid-treated group (prednisolone 15 mg/d) and a placebo group. Mortality rate was 14% in the treated group, and 56% in the placebo group, with a follow-up ranging from 30 to 72 mo. The trial from the Mayo Clinic published one year later^[23] included 63 patients, divided into four groups. Two groups were treated with protocols similar to current guidelines: the first group was treated with prednisone alone starting with 60 mg/d, tapered to a maintenance dose of 20 mg/d over 4 wk, the second group received prednisone 30 mg/d tapered to a maintenance dose of 10 mg/d combined with azathioprine at a fixed dose of 50 mg/d. The remaining groups were treated with azathioprine alone 100mg/d and placebo, respectively. The mortality rate in the first and second group was very low (6% and 7%), compared to a mortality rate of 36% and 41% in the groups treated with azathioprine

alone or placebo. The follow-up period ranged from 3 mo to 3.5 years. The side effect rate was lower in the azathioprine-prednisone group than in the prednisone alone group (10% vs 44%). A trial from King's College Hospital published in 1973^[24] included 47 patients, divided into two groups, one treated with prednisone 15 mg/d, and the other with azathioprine alone, 75 mg/d, with a follow-up of two years. The mortality rate in the prednisone group was 5%, as compared to a mortality rate of 24% in the azathioprine group. From these early trials it is clear that prednisone is very effective in treating AIH, and that azathioprine alone is not able to obtain disease remission. Following these reports, strategies were sought to optimize the treatment schedule, *i.e.* to find the minimal doses of prednisone or prednisone/azathioprine able to control the disease with minimal side effects. A trial published in 1975^[26] included 120 patients and compared four different schedules: (1) prednisone starting at 60 mg/d tapered to a maintenance dose of 20 mg/d; (2) prednisone starting at 30 mg/d tapered to 10 mg/d together with a 50 mg/d fixed dose of azathioprine; (3) prednisone at 60 mg/d tapered to a maintenance dose of 10 mg/d given on alternate days; and (4) placebo or azathioprine on a fixed dose of 100 mg/d without steroids, as control. Biochemical remission was achieved in 80% of patients in the first two groups, in 74% in the third group and in 34% in the control group. Histological remission was achieved in 57% and 60% of patients in the first two groups, but in only 19% and 24% in the third and in the control group. Side effects were less frequent in patients treated with prednisone/azathioprine from disease presentation, for which a lower dose of prednisone was used, leading to the conclusion that combined treatment is preferable. Of note, this trial enrolled "post-pubertal subjects", including patients from the age of 12 years. An additional trial published in 1982^[27] compared a fixed low-dose prednisone alone (10 mg/d for body weight < 70 kg, 15 mg/d for body weight ≥ 70 kg) in 37 patients with a fixed low-dose azathioprine alone (5 mg/kg per week for the first 2 wk, subsequently 10 mg/kg per week) in 47 patients. Mortality was very high in both groups at 1 year (27% and 28% respectively), indicating that a low prednisone dose and azathioprine alone are inadequate.

Despite the limitations of these early trials, prednisone ± azathioprine remains the mainstay of treatment for AIH, several reports showing high remission rates and favourable outcomes in both adult and juvenile AIH^[20,28-38].

Of note, azathioprine monotherapy, though unsuccessful in the induction of remission, is effective in adults as maintenance therapy, at a dose of 2 mg/kg per day^[39]. A 5-patient report suggests that it may be effective also in children^[40]. In a recent retrospective series, 87% of 66 children with AIH were reported to maintain sustained biochemical remission (normal transaminase levels) in association with low 6-thioguanine nucleotides (TGN) levels (50-250 pmol

8 x 10 red blood cell cont) on an azathioprine dose of 1.2-1.6 mg/kg per day with or without associated steroids^[41].

How to use prednisone and azathioprine

There is no treatment schedule applicable to all AIH patients. The suggested algorithms and treatment schedules must be tailored to the single patient, taking into account the severity of the disease, age and comorbidities^[1].

The AASLD guidelines published in 2010^[2] recommend two alternative schedules: either prednisone alone at a dose of 60 mg/d or a combination of prednisone 30 mg/d and azathioprine 50 mg/d as initial treatment, favouring the latter because of fewer steroid side-effects^[26]. However, as azathioprine can be hepatotoxic, particularly in cirrhotic and jaundiced patients^[25], the more recent guidelines by the European Association for the Study of the Liver (EASL) recommend that it is added after two weeks of steroid monotherapy [prednisone 1 mg/kg per day in adults], when partial disease control has been achieved^[1]. In addition, this approach avoids the problem of distinguishing between azathioprine-induced hepatotoxicity and non-response, this distinction being an important issue in clinical practice. A retrospective series of 133 adult patients reports better results with a combination of steroids and another immunosuppressant (azathioprine in 96%, other unspecified drugs in 4%) from disease presentation compared to steroids alone or steroids followed by the addition of azathioprine/other immunosuppressants. Of note, only 2% of the patients included in this study were jaundiced at presentation^[42], possibly explaining the high remission rate on azathioprine, without hepatotoxicity.

Prednisone should be rapidly tapered (Table 1) to minimise steroid side effects. This rapid decrease of the prednisone dose requires weekly checks of the transaminase levels to monitor response. Azathioprine should be added if the transaminase levels stop decreasing on steroid treatment alone (Table 1). Ultimately 85% of the patients will need azathioprine in addition to low-dose prednisone^[12]. This protocol was originally used for children^[25], but it is suitable to treat adult patients as well, because it allows to avoid azathioprine in a small proportion of patients and especially because it limits steroid side effects, which are often the reason for non-adherence. The initial recommended dose of azathioprine in adults is 50 mg/d or 1 mg/kg per day^[2]. If steroid side effects are severe and require steroid discontinuation, the azathioprine dose is increased to 2 mg/kg per day.^[39,43]

In children, the recommended treatment schedule is similar to that of adults, but a higher steroid dose is required due to the more aggressive disease course in this age group (Table 2). Children were included in early clinical trials^[22,26], but a sub-analysis of paediatric patients was not performed, and the numbers were small. Current recommendations are based on series

from large centres, which report a remission rate of about 90% using predniso(lo)ne \pm azathioprine^[20,35,36]. Conventional treatment of juvenile AIH consists of prednisolone (or prednisone) 2 mg/kg per day (maximum 60 mg/d), decreased over a period of 4 to 8 wk in parallel to the decline of transaminase levels, to a maintenance dose of 2.5-5 mg/d (Table 2). Long-term low daily doses are not associated with impaired adult height^[44]. The timing for the addition of azathioprine as a steroid-sparing agent varies according to the protocols used in different centres. In some, azathioprine is added only in the presence of steroid adverse effects, or if the transaminase levels stop decreasing on steroid treatment alone. In other centres azathioprine is added after a few weeks of steroid treatment in all patients, when the serum aminotransferase levels begin to decrease. Some centres use a combination of steroids and azathioprine from the beginning, but caution is recommended because of the azathioprine hepatotoxicity mentioned above^[1,2,34]. The initial recommended azathioprine dose is 0.5 mg/kg per day^[12], which can be increased to 1-2 mg/kg per day until normalization of the transaminase levels is reached. As in adults, azathioprine alone has been shown to be able to control the disease as long-term maintenance therapy, although only in retrospective series^[40,41,45].

As AIH is very sensitive to prednisone, a maintenance dose of 5 mg/d is effective in controlling the disease, usually with, but sometimes without, azathioprine. Steroid reduction below 5 mg/d (or 2.5 mg/d in children) requires careful monitoring of transaminase levels, even if implemented after long-term disease remission. The dose should be reduced very slowly, *e.g.*, by 1 mg per month if 1 mg prednisone tablets are available, or, if not available, by reducing to 5-2.5 mg on alternate days for 1-2 mo, and then to 2.5 mg/d.

Side effects of steroids and azathioprine

Steroid side effects are dose and time dependent, and arise if a dose exceeding 7.5-10 mg/d is administered over several months^[25]. The most common side effect is the development of cushingoid features. In a retrospective monocentric study of 103 adult AIH patients^[46], mostly treated according to a standard protocol with a steroid starting dose of 1 mg/kg per day and a mean follow-up period of 95 mo, 15.5% developed cushingoid features. Although not severe, these changes are often a great concern for the patients, and may lead to non-adherence, with the dangerous consequence of poor disease control. Almost half of AIH patients discontinue steroids because of cosmetic changes (including acne) or obesity^[47]. Severe, but less frequent steroid side effects include osteoporosis, brittle diabetes, cataract, psychosis and hypertension^[2]. They are mainly related to the initial high dose, and are reversible^[43,46]. Monitoring of these

complications is advisable, including ophthalmologic controls and bone density scans on a regular basis.

Azathioprine side effects affect 10%-20% of patients and include hepatotoxicity, acute cholestatic hepatitis, pancreatitis, nausea and vomiting, rash, bone marrow suppression, veno-occlusive disease, opportunistic infections, and malignancy^[2]. The most common side effect is bone marrow suppression, which is unpredictable, and can be aggravated by concomitant cytopaenia due to liver disease and hypersplenism. Haematological monitoring is necessary, particularly at the beginning of treatment. Measurement of erythrocyte concentrations of thiopurine methyltransferase (TPMT) activity may be advisable before institution of azathioprine therapy, but does not invariably predict response to the drug or toxicity^[48,49]. TPMT genotyping predicts azathioprine haematological toxicity in those rare individuals with variant homozygosity, while heterozygotes do not experience more toxicity than wild-type patients^[50]. Five percent of patients develop early intolerance, most frequently with nausea and vomiting.

A possible complication of long-term treatment with azathioprine is the development of malignancies. In one study aiming at investigating disease control by azathioprine monotherapy at a dose of 2 mg/kg per day, 5 of 72 patients (7%) developed malignancies over a median follow up of 12 years^[39]. Recently, two cases of T-cell lymphoma in adolescents treated with azathioprine for AIH were reported^[51]. Thus, a lower azathioprine dose in association with low-dose steroids may be preferable for long-term maintenance therapy. Azathioprine is considered to be safe in pregnancy^[52-54].

Measurement of the azathioprine metabolites 6-TGN and 6-methylmercaptopurine can be helpful in identifying drug toxicity and non-adherence, and in distinguishing azathioprine hepatotoxicity from disease non-response, as shown by a retrospective study in adults^[55], and a small prospective study in children^[56], but an ideal therapeutic level of the 6-thioguanine metabolites has not been established for AIH, unlike for inflammatory bowel diseases (IBD).

Treatment withdrawal

The AASLD^[2] and the EASL guidelines^[1] recommend a treatment duration of at least 2 and 3 years respectively, and both advise against a trial of treatment withdrawal before 2 years of complete biochemical remission. They recommend performing a liver biopsy before attempting treatment discontinuation, because histological inflammatory activity can still be present despite biochemical remission, predicting relapse. A recent report on 28 patients in whom treatment was withdrawn without histological evaluation, shows that the 54% of patients who did not relapse had transaminase levels less than half the ULN and IgG levels below 12 g/L on low-dose monotherapy (azathioprine/mercaptopurine or steroids) for at

least 2 years, suggesting that patients meeting these parameters may avoid pre-withdrawal liver biopsy^[57]. This suggestion, however, requires confirmation by other centres.

Relapse after treatment withdrawal is frequent, having been reported in some 80% of patients^[1,2,58]. Repeated relapses are associated with a poorer prognosis and a higher rate of drug side effects^[2]. For this reason, patients experiencing a first relapse episode after appropriate evaluation of disease remission, should undergo life-long low-dose immunosuppressive therapy^[1].

For AIH type 2, relapse is almost universal if treatment is completely withdrawn^[20], and long-term low-dose maintenance therapy should be planned from the diagnosis. Since this condition mostly affects children, adolescents and young adults, life-long duration of the therapy should be discussed and carefully explained to the patients and their family.

ALTERNATIVE TREATMENTS

For patients who experience azathioprine side effects, ranging from the relatively frequent early gastrointestinal intolerance to the rarer and more serious bone marrow suppression, and for poor responders to standard treatment, alternative regimens are needed, primarily to avoid high-dose steroid side-effects. A systematic review of the published clinical data on pharmacological treatments different from prednisone and azathioprine is provided in this section. Treatments for whom there are only anecdotal data are not discussed cyclophosphamide^[59], methotrexate^[60-62], ursodeoxycholic acid^[63-69], etanercept^[70], plasma exchange^[71], intravenous immunoglobulin^[72], leukapheresis^[73], chloroquine^[74], thymostimulin^[75], deflazacort^[76,77], saireito^[78], sympathomimetic amines^[79], glycyrrhizin^[80], fenofibrate^[81].

Budesonide

Budesonide is a glucocorticosteroid with a potent topical effect and a high first-pass uptake (> 90%) in the healthy liver, thus appearing ideal for treating AIH. The first reports on its use included small numbers of patients at different stages of disease and gave controversial results^[79-83] (Table 3). Subsequently, a large randomized controlled trial in 203 AIH patients (including 46 children/adolescents) was carried out, involving several European centres^[82] (Table 3). Cirrhotic patients were excluded, because the first pass hepatic extraction of budesonide may be reduced in cirrhosis due to portosystemic shunting. In fact, severe complications have been reported in cirrhotic patients on budesonide^[83,84], including portal vein thrombosis and Budd-Chiari syndrome, indicating that AIH patients with cirrhosis at diagnosis (at least one third) should not be treated with budesonide. The trial primary end-point was biochemical remission (defined as normalization of transaminase levels) in

absence of steroid side effects. The overall results of the trial showed better response to budesonide/azathioprine than to prednisone/azathioprine treatment, the primary end-point being achieved in 60% of patients given budesonide vs 38.8% of those given prednisone^[82]. These response rates, however, are below the remission rates achieved with standard treatment, and this has raised concerns. In the control arm, the prednisone dose was reduced as per-protocol, irrespective of the course of the clinical and biochemical response, an approach not recommended in AIH treatment^[1], which should be tailored to individual patient response. The initial prednisone dose (40 mg/d) was low at least for children/adolescents^[1,12], who should be treated with 2 mg/kg per day (up to 60 mg/d). All patients were prescribed azathioprine from the beginning, irrespective of the presence of jaundice, raising the possibility that the low response rate might be partly due to azathioprine hepatotoxicity^[85]. The trial included treatment naïve patients and patients experiencing disease relapse, who are likely to represent a subgroup of poor responders^[85]. Moreover, only transaminase levels were used to define biochemical remission, while the combination of normal transaminase and IgG/gammaglobulin levels best predicts absence of histological activity^[46,86,87].

Though budesonide is still not recommended as first line therapy for AIH^[1], it may be a valid alternative for the maintenance of remission long-time, particularly for patients experiencing steroid side effects. In a retrospective study 60 patients with either prednisolone side effects or dependence on a relative high dose of prednisolone were switched to budesonide^[88]: the biochemical remission rate at 6 mo was 55%, and 25% of the patients needed to be switched back to prednisone due to budesonide side-effects or insufficient response. However, all patients who were in remission at the time of switching remained in remission. These findings indicate that budesonide is effective in maintaining remission in patients who have achieved it with prednisone, but also that it is not free of side effects, and that, not surprisingly, it is not effective in patients resistant to prednisone, as prednisone and budesonide share the same receptor.

A sub-analysis of the paediatric population (46 patients aged 9 to 17) enrolled in the budesonide trial^[89] reported no significant difference in biochemical remission rate at 6 and 12 mo between the budesonide and the prednisone groups (32% and 33% at 6 mo and 50% and 42% at 12 mo, respectively) (Table 4). The frequency of steroid side effects was also not different, being 47% in the budesonide group and 63% in the prednisone group, apart from a lower mean weight gain in the budesonide group. The remission rate was well below that achieved with standard treatment, therefore, budesonide cannot be recommended for the treatment of children/

Table 3 Published data on autoimmune hepatitis treatment different from steroids and azathioprine in adults (from age 16)

Reference, yr	Country	Number and type of patients	Design	Outcome	Follow-up	Dose	Safety
Budesonide Danielsson <i>et al</i> ^[79] , 1994	Sweden	13 naïve	Prospective	Significant decrease of mean transaminase levels	9 mo	6-8 mg/d	Plasma cortisol reduction in cirrhotic patients
Czaja <i>et al</i> ^[80] , 2000	United States	10 AZA-NR	Prospective	3/10 BR	2-12 mo	9 mg/d	All patients had side-effects
Wiegand <i>et al</i> ^[81] , 2005	Germany	12 naïve	Prospective	10/12 BR	3 mo	9 mg/d	3 discontinued due to side effects
Csepreghi <i>et al</i> ^[82] , 2006	Germany	10 naïve	Prospective	7/10 naïve BR	24 wk	9 mg/d	Steroids side-effects in cirrhotic patients
Zandieh <i>et al</i> ^[83] , 2008	Canada	6 AZA-INT 3 PDN-INT	Retrospective	4/6 AZA-INT CBR 3/3 PDN-INT CBR	24 wk-8 yr	1.5-9 mg/d	Not reported
Manns <i>et al</i> ^[84] , 2010 ¹	Europe	208 naïve or relapsing	Prospective, randomized,	60% BR in budesonide 39% BR in PDN	6 mo	9 mg/d	Steroids side effects: 28% in budesonide arm, 53% in PDN arm
Mycophenolate mofetil Richardson <i>et al</i> ^[177] , 2000	United Kingdom	3 AZA-INT 4 AZA-NR	Retrospective	5/7 BR	46 mo	2 g/d	Leukopaenia in 1
Zolfino <i>et al</i> ^[93] , 2002	United Kingdom	3 second line	Retrospective	1/3 BR	Not reported	2 g/d	Not reported
Devlin <i>et al</i> ^[94] , 2004	Canada	5 second-line	Retrospective	5/5 BR	Not reported	Not reported	1 pyelonephritis
Chatur <i>et al</i> ^[95] , 2005	Canada	11 second-line	Retrospective	7/11 BR	10-54 mo	0.5-2 g/d	Leukopaenia in 1, diarrhoea in 1
Czaja <i>et al</i> ^[96] , 2005	United States	8 first- and second line	Retrospective	0/8 CBR	12-26 mo	0.5-3 g/d	None reported
Inductivo-Yu <i>et al</i> ^[97] , 2007	United States	15 second-line	Retrospective	Significant decrease of mean transaminase levels and of histological fibrosis and inflammation	41 mo	2 g/d	None significant
Hlivko <i>et al</i> ^[98] , 2008	United States	17 naïve 12 second-line	Retrospective	16/19 BR	Not reported	0.5-2 g/d	10 discontinued for side-effects
Hennes <i>et al</i> ^[99] , 2008 ²	Germany	27 AZA-INT 9 AZA-NR	Retrospective	57% AZA-INT BR 25% AZA-NR BR	16 mo	1-2 g/d	11 GI side effects
Wolf <i>et al</i> ^[178] , 2009	United States	16 second-line	Retrospective	5/16 BR	Not reported	1-2 g/d	1 discontinued due to paresthesias
Sharzei <i>et al</i> ^[100] , 2010	United States	9 AZA-INT 12 AZA-NR	Retrospective	21/21 BR	12 mo	0.5-2 g/d	1 discontinued for GI side-effects
Baven-Prongk <i>et al</i> ^[101] , 2011	The Netherlands	23 AZA-INT	Retrospective	67% AZA-INT BR 13% AZA-NR BR	3-133 mo	0.5-3 g/d	6 discontinued for side-effects
Jothinami <i>et al</i> ^[102] , 2014	India- United Kingdom	18 AZA-INT 2 AZA-NR	Retrospective	14 BR	5-83 mo	1-2 g/d	3 discontinued due to side-effects
Zachou <i>et al</i> ^[103] , 2016	Greece	109 naïve	Prospective	83/102 BR at 3 mo	72 mo	1.5-2 g/d	2 discontinued for septicaemia; 5 dose reduction for leukopaenia or infections
Gazzola <i>et al</i> ^[179] , 2016	Australia	51 AZA-INT 45 AZA-NR	Retrospective	27/49 AZA-INT BR 17/40 AZA-NR BR	Median: 31.9 mo	1-2 g/d	1 death, 2 hospitalisations, 8 GI side effects, 5 infections, 3 cytopoenia, 3 neuropsychiatric, 2 skin cancer, 1 lymphoproliferative disorder
Park <i>et al</i> ^[180] , 2016	South Korea	1 AZA-INT	Retrospective	1/1 CBR	1 yr	1 g/d	None
Cyclosporine A Mistilis <i>et al</i> ^[121] , 1985	Australia	1 AZA-INT	Retrospective	1/1 BR	1 yr	Not reported	None
Paroli <i>et al</i> ^[116] , 1992	Italy	3 naïve	Prospective	3/3 BR	1 yr	5 mg/kg/d	Not reported
Person <i>et al</i> ^[118] , 1993	United States	1 second-line	Retrospective	BR	Not reported	Not reported	Not reported
Sherman <i>et al</i> ^[114] , 1994	United States	6 AZA-NR (1 paediatric)	Retrospective	5/6 BR at 10 wk	Not reported	Not reported	1 increased serum creatinine
Senturk <i>et al</i> ^[119] , 1995	India	1 second-line	Retrospective	BR	1 yr	Not reported	None

Fernandes <i>et al</i> ^[113] , 1999	United States	5 AZA-NR	Retrospective	4/5 BR at 3 mo	27 mo	3-5 mg/kg/d	Minimal
Malekzadeh <i>et al</i> ^[117] , 2001	Iran	9 naïve	Prospective	79% BR and HI	26 mo	2-5 mg/kg/d	4 discontinued due to side effects
Zolfino <i>et al</i> ^[93] , 2002	United Kingdom	1 second-line	Retrospective	NR	Not reported	Serum level 100-200 µg/L	Not reported
Malekzadeh <i>et al</i> ^[181] , 2012	Iran	22 steroid-intolerant or NR	Retrospective	9 BR	60 mo	Not reported	Hirsutism (frequency not reported)
Tacrolimus							
Van Thiel <i>et al</i> ^[134] , 1995	United States	21 naïve	Prospective	Mean 80% ALT drop at 3 months	3 mo	6.6-8 mg/d; blood levels 0.6-1.0 ng/mL	Mild mean creatinine elevation after 1 yr
Heneghan <i>et al</i> ^[137] , 1999	United Kingdom	7 naïve	Prospective	BR in 86%		Not reported	Not reported
Zolfino <i>et al</i> ^[93] , 2002	United Kingdom	5 AZA-NR	Retrospective	2/5 BR	Not reported	2-4 mg/d	Not reported
Aqel <i>et al</i> ^[130] , 2004	United States	11 second-line	Retrospective	Normalization of mean ALT value	16 mo	0.5-1 mg/d (blood level < 6 ng/mL)	Minimal
Chatur <i>et al</i> ^[95] , 2005	Canada	3 second-line	Retrospective	3/3 NR	10-54 mo	2-4 mg/d	1 discontinued for abdominal pain
Larsen <i>et al</i> ^[131] , 2007	Denmark	9 AZA- or MMF-NR (1 pediatric)	Retrospective	9/9 BR	12-37 mo	2 mg/d (target blood level < 6 ng/mL)	1 mild tremor
Tannous <i>et al</i> ^[133] , 2011	United States	13 second-line	Retrospective	12/13 BR	1-65 mo	2-6 mg/d (mean blood level 6 ng/mL)	1 HUS; 1 oral carcinoma
Than <i>et al</i> ^[135] , 2016	German, United Kingdom	16 AZA-NR 1 AZA-INT	Retrospective	BR in most	60 mo	0.5-5 mg/d	1 LT; 4 PSC overlap
Al Taii <i>et al</i> ^[136] , 2017 ³	United States	23 second-line	Retrospective	27% CBR 41% BR		5 mg/d (mean serum level: 6.7 ng/mL (mean))	Significant increase of serum creatinine; 1 discontinued for GI hemorrhage
Sirolimus							
Chatrath <i>et al</i> ^[139] , 2014	United States	5 AZA-NR	Prospective	4/5 BR	4-72 mo	2 mg/d	2 hyperlipidemia
Rubin <i>et al</i> ^[141] , 2016	United States	2 second-line	Retrospective	1/2 BR	Not reported	3-6 mg/d	1 discontinued due to leg ulcer
Everolimus							
Ytting <i>et al</i> ^[143] , 2015	Denmark	7 second-line	Retrospective	3/7 CBR 4/7 BR	1-3 yr	0.75-1.5 mg/d (target blood levels: 3-6 ng/mL)	Minimal
Rituximab							
Burak <i>et al</i> ^[144] , 2013	Canada	3 AZA-NR 3 AZA-INT	Prospective	6/6 BR at 24 wk	72 wk	1000 mg on day 0 and 15	1 mild infection
Al-Busafi <i>et al</i> ^[182] , 2013	Oman	1 steroid-resitant	Retrospective	BR	Not reported	Not reported	None reported
Rubin <i>et al</i> ^[141] , 2016	United States	1 second-line	Retrospective	1/1 BR	14 mo	475 mg/m ² per week	None reported
Infliximab							
Weiler-Normann <i>et al</i> ^[154] , 2013	Germany	11 second-line	Retrospective	8/11 BR	6 to > 40 infusions	5 mg/kg on 0, 2, 6, then every 4-8 wk	7/11 infections, 3 discontinued for side effects
Vallejo <i>et al</i> ^[156] , 2014	Spain	1 AZA-NR	Retrospective	1/1 BR	3 mo	5 mg/kg given 3 times	Mild respiratory infection
6-mercaptopurine							
Pratt <i>et al</i> ^[167] , 1996	United States	2 AZA-INT	Retrospective	2/2 CBR, 1/2 HI	24 mo in one not reported in the other	100 mg/d	None reported
Hübener <i>et al</i> ^[168] , 2016	Germany/United Kingdom	20 AZA-INT 2 AZA-NR	Retrospective	8/20 CBR 7/20 BR	18.5 mo	25-100 mg/d	4 discontinued for GI side-effects, 1 for leucopaenia
Elnegouly <i>et al</i> ^[183] , 2017	Germany/Austria	17 AZA-INT	Retrospective	11/12 CBR	1 yr	25-50 mg/d	2 discontinued for side-effects
Allopurinol							
Al-Shamma <i>et al</i> ^[170] , 2013	United Kingdom	1 AZA-NR	Retrospective	1/1 BR	12 mo	100 mg/d	None reported

De Boer <i>et al</i> ^[171] , 2013	The Netherlands	3 AZA-INT 5 AZA-NR	Retrospective	7/8 BR	13 mo	100 mg/d	1 discontinued for neuropathy
Al-Shamma <i>et al</i> ^[172] , 2013	United Kingdom	1 AZA-NR	Retrospective	1/1 CBR	Not reported	100 mg/d	None reported
6-thioguanine							
De Boer <i>et al</i> ^[174] , 2005	The Netherlands	3 AZA-INT	Retrospective	3/3 BR	Not reported	0.3 mg/kg/d	None reported
Van den Brand <i>et al</i> ^[175] , 2017	The Netherlands	6 AZA-NR 6 AZA-INT	Retrospective	Significant median ALT decrease	12-75 mo	0.3 mg/kg/d	1 nodular regenerative hyperplasia

¹The series includes 46 children (Woynarowski *et al*^[91] 2013); ²The series includes 4 adolescents, but only overall results are reported, and youngest age at diagnosis was 13 yr; ³The series includes 6 adolescents, but only overall results are reported, and youngest age at diagnosis was 15 yr. BR: Biochemical response; AZA-NR: Azathioprine non-responder; AZA-INT: Azathioprine intolerant; CBR: Complete biochemical response; PDN: Prednisone; GI: Gastrointestinal; HI: Histological improvement; LT: Liver transplant; NR: Non-responder; ALT: Alanine aminotransferase; HUS: Haemolytic-uremic syndrome; PSC: Primary sclerosing cholangitis.

Table 4 Published data on autoimmune hepatitis treatment different from steroids and azathioprine in children

Ref.	Country	Number and type of patients (n)	Design	Outcome	Follow-up	Dose	Side effects
Budesonide							
Woynarowski <i>et al</i> ^[91] , 2013	Europe	46 including naïve and second-line	Prospective	16% BR AZA+BUD 15% BR AZA+PDN at 6 mo	1 yr	6-9 mg/d	More weight gain in PDN group
Mycophenolate mofetil							
Lee <i>et al</i> ^[107] , 2007	Malaysia	2 second-line	Retrospective	0/2 BR at 6 mo	6-18 mo	20-40 mg/kg/d	Not reported
Aw <i>et al</i> ^[106] , 2009	United Kingdom	20 AZA-NR 6 AZA-INT	Retrospective	18/26 CBR	0.75-12 mo	20-40 mg/kg/d	7 Leukopenia
Jiménez-Rivera <i>et al</i> ^[108] , 2012	Canada	12 second-line	Retrospective	Not reported	Not reported	1000-1500 mg/d	Not reported
Dehghani <i>et al</i> ^[109] , 2013	Iran	5 second-line	Retrospective	5/5 BR	None reported	Not reported	Not reported
Cyclosporine A							
Jackson <i>et al</i> ^[120] , 1995	South Africa	1 AZA-INT	Retrospective	1/1 BR at 2 wk	19 mo	5 mg/kg/d	None
Debray <i>et al</i> ^[111] , 1999	France	8 naïve 7 second-line (all type 2 AIH)	Retrospective	8/8 naïve BR 7/7 second-line (including 3 with ALF)	1-6 yr	4.7-5.6 mg/kg/d	Minimal
Ben Halima <i>et al</i> ^[122] , 2002	Tunisia	1 first-line	Retrospective	1/1 BR	Not reported	Not reported	None
Sciveres <i>et al</i> ^[184] , 2004	Italy	4 naïve 4 steroid/AZA-intolerant	Retrospective	8/8 BR at 2-8 wk	1.5-15 yr	4-10 mg/kg per day	2 gingival hypertrophy, 1 creatinine elevation
Cuarterolo <i>et al</i> ^[124] , 2006	Argentina	86 naïve, type 1 AIH	Prospective	BR 94%	2 yr	4 mg/kg per day	8/84 creatinine elevation 3/84 hypertension 11 hypertrichosis, 13 gingival hypertrophy
Nastasio <i>et al</i> ^[115] , 2011	Italy	19 naïve ¹ 10 second-line ¹	Retrospective	19/19 naïve BR at 4-18 wk 9/10 second-line BR	6.5 yr	Not reported	
Dehghani <i>et al</i> ^[109] , 2013	Iran	3 second-line	Retrospective	3/3 BR	Not reported	Not reported	Not reported
Lee <i>et al</i> ^[107] , 2015	Malaysia	2 second-line	Retrospective	1 / 2 BR	6-18 mo	5 mg/kg per day, serum level 250-350 ng/mL	
Zaya <i>et al</i> ^[112] , 2012	Croatia	9 naïve (1 type 2 AIH)	Retrospective	7/9 BR after 1 yr	24 mo	3-5 mg/kg per day	Minor
Jiménez-Rivera <i>et al</i> ^[108] , 2012	Canada	9 naïve 15 second-line	Retrospective	Not reported	4 ± 2 yr	4 ± 0.8 mg/kg per day initially 4.9 ± 1.8 mg/kg per day in follow-up	Not reported
Tacrolimus							
Zolfino <i>et al</i> ^[93] , 2002	United Kingdom	1 second-line	Retrospective	NR	Not reported	2 mg/d	Not reported
Marlaka <i>et al</i> ^[138] , 2012	Sweden	20 naïve	Prospective	3/20 BR in monotherapy	1 yr	Target blood levels: 2.5-5 ng/ml	1 discontinued for side-effects; 2 developed IBD
Dehghani <i>et al</i> ^[109] , 2013	Iran	2 second-line	Retrospective	2/2 BR	Not reported	Not reported	Not reported

Jiménez-Rivera <i>et al</i> ^[108] , 2015	Canada	6 second-line	Retrospective	Not reported	Not reported	Not reported	Not reported
Sirolimus							
Kurowski <i>et al</i> ^[140] , 2014	United States	4 second-line	Retrospective	2/4 BR	Not reported	Not reported	2 mo ulcers
Rituximab							
D'Agostino <i>et al</i> ^[150] , 2013	Canada/Argentina	2 second-line	Retrospective	2/2 CBR at 3/8 mo	26-38 mo	375 mg/m ² weekly for 4 wk	None reported
Infliximab							
Rajanayagam <i>et al</i> ^[158] , 2013	Australia	1 second-line	Retrospective	1/1 BR	19 mo	5 mg/kg 4 infusions at 4 wk interval	LT was not prevented
6-mercaptopurine							
Pratt <i>et al</i> ^[167] , 1996	United States	1 AZA-NR	Retrospective	1/1 CBR and HR	36 mo	1.5 mg/kg	None reported

¹Twelve patients had additional concomitant immunosuppressive drugs. BR: Biochemical response; AZA: Azathioprine; BUD: Budesonide; PDN: Prednisone; INT: Intolerant; NR: Non-responder; AIH: Autoimmune hepatitis; ALF: Acute liver failure; IBD: Inflammatory bowel disease; LT: Liver transplant; CRB: Complete biochemical response.

adolescents with AIH until a trial including strict diagnostic criteria and drug schedules appropriate for the juvenile disease is performed^[85].

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is the prodrug of mycophenolic acid. It is an inhibitor of inosine monophosphate dehydrogenase, the rate-limiting enzyme in de novo purine synthesis on which, in contrast to other cells, B and T lymphocyte proliferation relies. MMF is widely used as second line AIH treatment, mostly combined with prednisone, both for patients intolerant to azathioprine and for patients with unsatisfactory response to standard azathioprine/prednisone treatment. Its use in AIH is based on retrospective series^[90-103] (Table 3) with a total number of 313 patients treated, suggesting that MMF is partially effective in patients intolerant to azathioprine, but may not be effective in case of azathioprine poor response. However, a recent paper from Australia including 96 patients^[104] reported a similar remission rate both in patients intolerant and poor responders to azathioprine (Table 3). One single prospective uncontrolled trial from Greece tested the use of MMF as first-line treatment^[102,105] (Table 3). MMF was reported to be safe and effective in inducing and maintaining remission in treatment-naïve patients (83/102 patients achieved biochemical remission at 3 mo) and to have a rapid steroid sparing effect. However, it is not clear whether it offers an advantage over azathioprine, as a head-to-head comparison with azathioprine was not performed. A trial comparing azathioprine to MMF is currently ongoing (NCT02900443). MMF has the major disadvantages of being about 15 times more expensive than azathioprine, and, most importantly, of being teratogenic, which is highly relevant, since AIH affects mainly young females. The most frequent side effects are gastro-intestinal symptoms.

In juvenile AIH patients in whom standard immunosuppression is unable to induce stable remission, or who are intolerant to azathioprine, MMF at a dose of 20 mg/kg twice daily, together with prednisolone,

has been used successfully used^[90,106-108] (Table 4). A recent meta-analysis, including data from several small studies of second line treatments in children refractory to standard therapy shows that MMF is efficacious with a low side effect profile (in contrast to calcineurin inhibitors), supporting the notion that MMF should be the primary choice for second-line therapy in juvenile AIH^[109].

Calcineurin inhibitors

Cyclosporine A: Cyclosporine A is a calcineurin inhibitor extensively used in the setting of transplant medicine. Important side effects are renal toxicity and cosmetic changes, particularly in association to high doses. In small retrospective series^[90,108,110-115], small prospective open and uncontrolled trials^[115,116] and single case studies^[91,107,117-121] cyclosporine A has been reported to be effective - using variable doses, duration of treatment and follow-up - either as first-line option or in patients not responding to azathioprine and prednisone, both in children and in adults (Tables 3 and 4). Though the results of these reports appear to be encouraging, the quality and quantity of the data are insufficient to recommend its use. In paediatrics, cyclosporine A has been used as first line treatment for type 1 AIH in an attempt to reduce steroid side effects in a prospective multicentre study in 84 treatment-naïve children^[122,123] (Table 4). Cyclosporine alone was administered for 6 mo, and the patients were subsequently switched to azathioprine and prednisone. Transaminase levels normalization was obtained in 72% of the subjects after six months of cyclosporine monotherapy, but IgG levels were not included in the remission criteria. Cyclosporine side effects included hypertrichosis (55%), gingival hyperplasia (39%), elevation of creatinine (9%) and hypertension (3%). The main limitation of this study is lack of direct comparison with standard treatment.

Animal data suggest that cyclosporine A may promote autoimmunity^[124-127], and the first reports of de novo autoimmune hepatitis arising after liver transplantation were in children treated with

cyclosporine^[128]. These observations call for caution in the use of cyclosporine in AIH.

Tacrolimus: Tacrolimus is a more potent calcineurin inhibitor than cyclosporine, has less cosmetic side effects, but similar drug class toxicity. In AIH, it has been used both for refractory cases and for patients intolerant to other immunosuppressive regimens. A few retrospective small case series in adults have been published, with variable remission criteria, sometimes including only transaminase levels^[91,93,129-135]. The reported efficacy was good in a total number of 80 patients (Table 3). Two prospective open-label trials from the '90s are available, both in naïve patients^[133,136] (Table 3). The oldest one included 21 adult patients^[133], with a follow up of 1 year, after which a liver biopsy was repeated, but histological results are not reported. Half of the patients were anti-LKM1 positive; tacrolimus was used as monotherapy. Of note, the serum target level of tacrolimus was low (0.6-1 ng/mL). The mean decrease of transaminase and bilirubin levels was satisfactory, but the remission rate is not reported. In terms of side effects, the mean creatinine value increased significantly after 1 year of treatment. The second prospective trial in naïve patients included seven adult subjects and used lower tacrolimus doses combined with 20 mg/d of prednisolone. Transaminase levels, albumin, bilirubin and prothrombin time significantly improved in 6/7 patients^[136].

In children, one prospective, single centre, open label trial including 20 treatment naïve patients is available: none was anti-LKM1 positive, follow up was 1 year, after which a liver biopsy was repeated^[137] (Table 4). Target tacrolimus blood levels were 2.5-5 ng/mL. 14/20 patients needed azathioprine and prednisone in addition to tacrolimus to achieve remission. Histological improvement of inflammation was seen in 12/14 cases. No effect on the renal function was observed. This trial suggests that tacrolimus as monotherapy is not effective in juvenile AIH, but could be considered as steroid/azathioprine sparing agent.

More high-quality data are needed, both in adults and children, to assess tacrolimus efficacy in AIH.

m-TOR inhibitors

Sirolimus: Sirolimus is a macrolide molecule acting by inhibiting the mammalian target of rapamycin (mTOR), a protein that modulates the proliferation and survival of activated lymphocytes. It is produced by the bacterium *Streptomyces hygroscopicus* and was isolated in 1972 on Easter Island (Rapa Nui). Sirolimus is used to prevent rejection in solid organ transplantation.

There is very limited experience in the use of this drug for poor responders to standard AIH treatment. Retrospective data on 5 adult patients with AIH refractory to prednisone, azathioprine and

mycophenolate are available^[138] (Table 3). Only transaminase levels were used to define remission, median follow up was 24 mo, the target serum level was low, 10-20 ng/dL. Complete remission was achieved in 2/5 patients. Side effects were limited to hyperlipidaemia occurring in 2/5 patients. In paediatrics, a small retrospective series reports the use of rapamycin in 5 cases refractory to standard treatment (3/4 also to MMF)^[139], including 1 case of non-adherence (Table 4). Two of the four patients showed an improvement in transaminase levels; tolerability was good, though 2/4 had mouth ulcerations not requiring drug discontinuation. The target sirolimus blood levels reported in the paper are 4-8 ng/mL. A report of two additional adult cases of difficult-to-treat AIH patients managed with sirolimus is even less encouraging: in one case sirolimus was discontinued due to legs ulcers, and in the other it was ineffective^[140]. No drug serum levels were reported.

In conclusion, data on sirolimus in difficult-to-treat AIH patients are scanty and rather disappointing.

In the transplant setting, sirolimus has been reported to be effective in difficult-to-treat de novo AIH or AIH recurrence^[141] in a small series of 6 paediatric patients. Three of them experienced infections while on sirolimus, including one case of colitis and fever leading to drug discontinuation.

Everolimus: Everolimus has a mechanism of action similar to sirolimus, and is used to prevent solid organ rejection, or at higher doses, as an anti-cancer drug. Only one report is available on the use of everolimus for the treatment of AIH. It is a retrospective series of 7 adult patients with insufficient response to standard or alternative treatments (budesonide, MMF, calcineurin inhibitors), or with severe treatment side-effects^[142] (Table 3). Everolimus target blood concentration was 3-6 ng/mL. Complete biochemical response was obtained in 3/7 patients after 5 mo, but all patients, except one who was non-adherent, had significant decrease in serum transaminase levels, allowing reduction of the steroid dose. Histology did not show disease progression in four patients treated for 3-5 years. No severe side effects were reported, but one patient died from cholangiocarcinoma diagnosed 6 mo after starting everolimus, though cancer was not considered to be associated with the drug. In conclusion, in view of the very few data available, the role of everolimus in the treatment of AIH remains to be explored.

Biologicals

Rituximab: Rituximab is a monoclonal chimaeric (murine/human) antibody that specifically binds the CD-20 antigen, a phosphoprotein expressed on the surface of B-lymphocytes, leading to B-cell depletion. It is approved for the treatment of non-Hodgkin lymphoma, rheumatoid arthritis and ANCA-

associated vasculitis. It has also been used recently as rescue treatment in refractory AIH. In a single-centre open-label pilot study in Canada, 6 AIH adult patients who had failed treatment with prednisone and/or azathioprine^[143] for intolerable side-effects (3/6) or refractory disease (3/6) were treated with two doses of 1000 mg rituximab administered two weeks apart (Table 3). Tolerance was good, only one patient developing minor infections. In all patients, transaminase and IgG levels decreased; a liver biopsy performed after 1 year in 4 of the 6 patients showed improvement of the inflammatory activity. Though a recent survey shows that rituximab is used for difficult-to-treat AIH patients in several centres^[144], this experience has not been published. A few case reports of patients with AIH coexisting with other autoimmune diseases have been published^[145-149], all demonstrating a positive effect of rituximab also on AIH.

In children, two cases of refractory AIH have been successfully treated with rituximab^[149] (Table 4). In addition, the recently published preliminary results of a real-world expert management of paediatric AIH also reported the use of rituximab as rescue therapy^[150].

In summary, rituximab has shown good efficacy in a small number of difficult-to-treat AIH patients, but its safety profile needs to be evaluated carefully, as the drug may have severe long term side-effects, including B-cell depletion^[151].

Infliximab: Infliximab is a recombinant humanized chimaeric antibody used for the treatment of ulcerative colitis, Crohn disease, rheumatoid arthritis, psoriatic arthritis/plaque psoriasis, and ankylosing spondylitis. It acts mainly by direct neutralization of soluble tumour necrosis factor- α , but it has also pro-apoptotic and anti-proliferative effects on lymphocytes^[152].

One small retrospective series from Germany on the use of infliximab as salvage therapy in 11 adult AIH patients reports^[153] (Table 3) normalisation of transaminase levels in 8 and of IgG levels in 6. However, 7 patients developed infectious complications, and treatment had to be stopped because of side effects in three cases. Recently, preliminary results of an extension of this cohort of difficult-to-treat AIH patients was published: the cohort now includes 18 cases, 15 reaching biochemical remission^[154]. Two case reports have also been published: one describing a difficult-to-treat AIH patient who achieved normalization of transaminases levels after 3 mo of infliximab treatment^[155], and one reporting good disease control on infliximab in a young patient with AIH and adult onset Still disease^[156].

In children, a 10-year old girl with aggressive disease, unresponsive to standard treatment, MMF and tacrolimus, has been reported to have a good response to infliximab, though liver transplantation was deferred but not avoided^[157] (Table 4).

As for rituximab, specialized centres have unreported experience^[144,150]. It is important to note that anti-tumour necrosis factor- α can induce hepatotoxicity resembling AIH^[158-162], as well as other immune-mediated disorders, such as lupus erythematosus^[163]. This should raise caution in using this agent, which should be reserved for treatment-resistant AIH cases in specialised centres.

Thiopurines

6-mercaptopurine: Azathioprine is the prodrug of 6-mercaptopurine (6-MP), and is non-enzymatically converted into 6-MP, which represents the biologically active form of the drug. 6-MP is used for the treatment of IBD, where it has been shown that 6-MP is better tolerated than azathioprine^[164,165], despite the close biochemical relationship and shared metabolic pathways (Figure 2). In AIH, 6-MP was used successfully in 3 patients intolerant or unresponsive to azathioprine, including one paediatric patient^[166], representing the only published experience in children (Tables 3 and 4). The largest series of AIH patients intolerant or unresponsive to standard treatment switched to 6-MP is a retrospective study on 22 adult cases^[167] (Table 3). The two patients with insufficient response to standard treatment did not respond to 6-MP, whereas 15/20 patients intolerant to azathioprine showed either partial (7/15) or complete (8/15) biochemical remission. Five patients discontinued 6-MP, four for gastrointestinal side effects, and one for leukopaenia. Recently, preliminary data from an additional multicentre retrospective series of 17 patients, all azathioprine-intolerant, reported complete biochemical response in 11 of the 12 patients followed-up for at least 12 mo^[168]. These data suggest that 6-MP can be an alternative for patients intolerant to azathioprine, but the available data are insufficient to formulate recommendations.

Allopurinol: Azathioprine hepatotoxicity can be due to a skewed metabolism of the drug, leading to a preferential generation of the hepatotoxic metabolite 6-methylmercaptopurine (6-MMP) instead of the metabolic active 6-thioguanine nucleotides (6-TGN). Allopurinol co-administration redirects the thiopurine metabolism towards 6-TGN. This strategy is used in the treatment of IBD. A case report suggests that allopurinol can be helpful also in AIH^[169] (Table 3). A retrospective case-series of 8 AIH adult patients intolerant or with insufficient response either to azathioprine/prednisone (4/8) or 6-MP/prednisone (4/8), one patient in each group being also on budesonide, reported complete biochemical remission in 3/3 intolerant patients and in 4/5 unresponsive patients^[170] (Table 3). All patients had skewed thiopurine metabolism. In one further case report of a patient with insufficient response to prednisone/

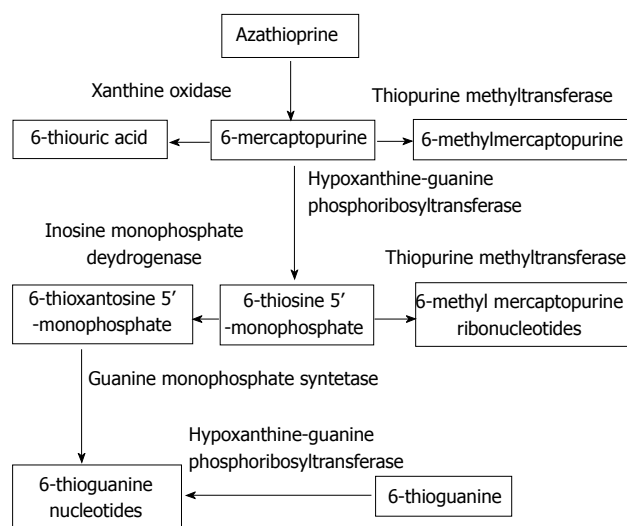


Figure 2 Simplified representation of the thiopurine metabolism. Azathioprine is non-enzymatically converted to 6-mercaptopurine, which is competitively converted into 6-methylmercaptopurine, 6-thiouric acid and 6-thiosine 5'-monophosphate by different enzymes. The latter metabolite is further transformed into the metabolic active 6-thioguanine nucleotides.

azathioprine and shunted metabolism, allopurinol (100 mg/d) allowed rapid normalisation of transaminase levels and steroid reduction^[171] (Table 3).

6-thioguanine: 6-thioguanine (6-TG) is enzymatically converted into 6-TGN, which are the active metabolites of azathioprine, bypassing the metabolic steps leading to the formation of the hepatotoxic metabolite 6-MMP (Figure 2). 6-TG is approved for the treatment of acute and chronic myeloid leukaemia, and chronic lymphatic leukaemia. It is used in IBD patients with insufficient response or intolerant to azathioprine or 6-MP^[172]. Safety issues have been raised, particularly in respect to the development of nodular regenerative hyperplasia and sinusoidal obstruction syndrome^[172]. In AIH, after an early preliminary report^[173], a retrospective series of 12 adult patients switched from azathioprine or 6-MP to 6-TG for intolerance or insufficient response reported a median alanine aminotransferase levels drop from 81 IU/L to 30 IU/L (Table 3). Nodular regenerative hyperplasia developed in one case after 8 years of 6-TG treatment^[174].

Due to the paucity of data and its potential hepatotoxicity, 6-TG cannot be recommended in AIH.

TREATMENTS UNDER INVESTIGATION

New compounds are currently under investigation in AIH. Preliminary results of a phase 1, first-in-human trial of preimplantation factor in AIH demonstrated good safety and tolerability, but a non-significant decrease in mean transaminase levels^[175]. Other investigational drugs in AIH include VAY736, which leads to B-cell depletion and B-cell activating factor receptor blockade (NCT03217422), JKB-122, which is

a toll-like receptor 4 antagonist (NCT02556372) and low dose interleukin 2 (NCT01988506).

CONCLUSION

The pharmacological treatment of AIH should be personalized, because of the heterogeneity of the disease. Treatment schedules in children differ, because of the more aggressive disease course in this age group. Standard treatment, based on steroids and azathioprine, is effective in the vast majority of patients, and side-effects can be minimised by rapid prednisone tapering. Budesonide was tried as first-line treatment in an attempt to reduce steroids side-effects, but the results of a randomized controlled trial do not allow to universally recommending it as first-line treatment instead of prednisone. A minority of patients prove difficult-to-treat, either because of severe side effects from standard treatment, or resistant disease. Mycophenolate mofetil is the most widely used second-line drug, and also the drug with the highest amount of available data. Calcineurin inhibitors are alternative options, but data on their efficacy are scanty. Infliximab and rituximab may represent an additional treatment option for selected difficult-to treat cases, but their use should be restricted to specialised centres because of potentially severe side effects. New pharmaceutical treatments are currently under investigation.

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Colorectal cancer, screening and primary care: A mini literature review

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Abstract

Colorectal cancer (CRC) is a common health problem, representing the third most commonly diagnosed cancer worldwide and causing a significant burden in terms of morbidity and mortality, with annual deaths estimated at 700000. The western way of life, that is being rapidly adopted in many regions of the world, is a well discussed risk factor for CRC and could be targeted in terms of primary prevention. Furthermore, the relatively slow development of this cancer permits drastic reduction of incidence and mortality through secondary prevention. These facts underlie primary care physicians (PCPs) being assigned a key role in health strategies that enhance prevention and prompt diagnosis. Herein, we review the main topics of CRC in the current literature, in order to better understand its pathogenesis, risk and protective factors, as well as screening techniques. Furthermore, we discuss preventive and screening policies to combat CRC and the crucial role served by PCPs in their successful implementation. Relevant articles were identified through electronic searches of MEDLINE and through manual searches of reference lists.

Key words: Colorectal cancer; Prevention; Diagnosis; Screening; Primary care

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Core tip: Colorectal cancer (CRC) is a common health problem, causing a significant burden in terms of morbidity and mortality. However, if detected early, the disease is highly curable. Primary care physicians are therefore in a unique position to enhance prevention and prompt diagnosis. The purpose of this paper was to

review the main topics of CRC in the current literature to provide a more comprehensive understanding of its pathogenesis, risk and protective factors, as well as screening techniques.

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INTRODUCTION

Colorectal cancer (CRC) is a global health burden, accounting for almost 700000 deaths per year worldwide^[1]. CRC is the third most commonly diagnosed cancer worldwide and second in Europe^[1]. According to the World Health Organization GLOBOCAN database, in 2012, almost 1.4 million new cases of CRC were diagnosed and almost 700000 deaths occurred worldwide^[1].

CRC global incidence and mortality rates appear to be substantially higher for males than for females, with 21 new cases and 10.5 deaths per 100000 population compared to 17.6 new cases and 9.2 deaths respectively. In males, CRC ranks third in incidence, following lung and prostate cancers, and in females it ranks second, following breast cancer^[1,2].

There is an over 10-fold geographical variation of CRC incidence throughout the world^[3]. The highest incidence rates are observed in Australia and New Zealand, with the estimated age-standardized rates being 44.8 per 100000 population in men and 32.2 in women; Europe and North America follow close behind. The lowest incidence is observed in Africa, with the rates in Western Africa being only 3.5 per 100000 in men and 3.0 in women^[1,3,4].

Incidence trends reported for the past few decades have revealed very interesting findings. In the United States, overall CRC incidence has been declining since the mid-80s, right about the time that CRC screening was introduced^[5-7]. In Europe, incidence trend patterns show great diversity among countries, mainly due to differences in screening policies and prevalence lifestyle risk factors between countries. The largest increase in incidence was observed in Central-Eastern Europe over the past few decades^[8-12].

CRC incidence increases with age, and cases are fairly uncommon before the 4th decade of life^[1,3]. This is the reason why most screening programs are targeted to people over 50 years old. Nevertheless, recent studies have revealed an alarming increase in incidence between the ages 40 to 44, prompting consideration of lowering the recommended screening age^[13,14].

Mortality rates have progressively declined in most economically developed countries, in contrast with poorer regions of the world, where mortality is either

stable or increasing^[1,11]. This reflects the diversity in screening services accessibility, specialized care and lifestyle risk factors^[11]. The highest reported mortality rates are in Central-Eastern Europe, although the highest incidence to mortality ratio is observed in Middle-Western Africa^[1,15].

In this paper, we aimed to perform a narrative literature review and compile all of the up-to-date knowledge on the current CRC medical literature. Our main objective was to summarize all the available information and provide gastroenterologists and primary care physicians (PCPs) with a comprehensive background for a better understanding of the current evidence.

SEARCH STRATEGY

We conducted a literature search in the PubMed database, with publication date limited to between January 1996 and August 2016, using the following Medical Subject Heading (commonly known as MeSH) terms: "colorectal neoplasms", "diagnosis", "early detection of cancer", "primary health care". The search was limited to English language. Editorials, Letters to the Editor and Case Reports were excluded. Inclusion criteria for papers were CRC topics in prevention, screening, detection and diagnostics, as well as follow-up in primary care. The titles and abstracts of all papers identified by the electronic search were manually assessed by two researchers working independently (AH, DA). Disagreements between the two reviewers were infrequent and resolved by consensus or arbitration of a third reviewer (MK).

Full texts of the articles that were considered eligible for inclusion were also scrutinized in order to offer a better approach on CRC issues related to pathogenesis, screening, diagnosis and management, as were articles related to early detection in the primary care setting.

A total of 159 studies were identified and assessed for eligibility. Among them, 7 overlapped and were excluded. Four articles were also excluded due to topical relevance to other types of cancer. Finally, 148 articles were assessed in detail for study inclusion. From these, 42 were excluded for not meeting the inclusion criteria. Figure 1 summarizes the process of identification and selection of studies.

PATHOGENESIS

The Adenoma-Carcinoma pathogenesis model is what gives endoscopic methods of screening the benefit of not only reducing mortality but also reducing incidence of CRC through early recognition and removal of adenomatous polyps from the colon^[16]. The Adenoma-Carcinoma sequence applies to most CRCs and involves a sequential progression that takes, on average, a decade to occur. Many of the adenomas begin as small polyps that enlarge and become

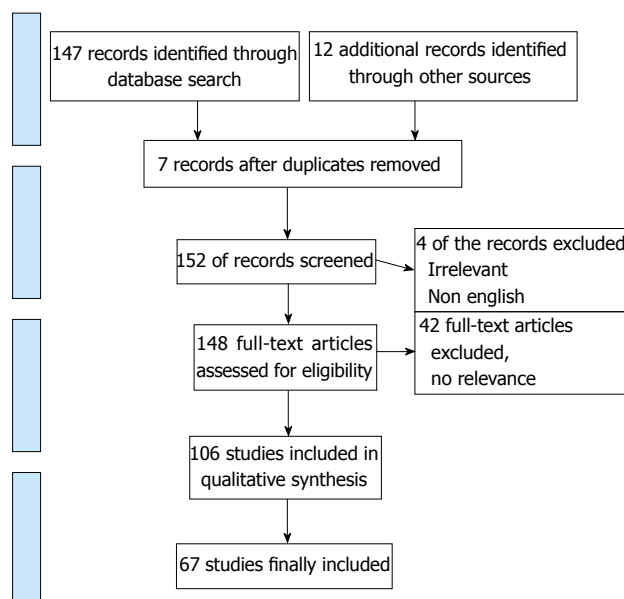


Figure 1 Study selection based on prisma diagram^[112].

dysplastic and eventually cancerous^[17-19]. Other CRCs may develop from non-polypoid adenomas, that are, by definition, more difficult to detect^[20]. Nevertheless, there have been studies that suggest alternative developmental pathways to CRC, other than the traditional aforementioned model^[21-23].

RISK FACTORS

The probability for developing CRC can be increased by both genetic and acquired/environmental factors. Although the impact of genetic susceptibility in an individual is much greater than the impact of acquired factors, the vast majority of CRC cases could be prevented through modifications in environmental factors^[24-26]. A constructive way of classifying CRC risk factors is separating those that affect screening recommendations from those that do not. Hereditary syndromes, family history and inflammatory bowel diseases are the main risk factors that affect recommendations for screening^[27]. In practice, risk factors that do not affect screening are the target of primary prevention strategies^[24].

Hereditary colorectal syndromes comprise numerous specific genetic disorders that are associated with the development of CRC, altogether accounting for about 10% of CRC cases. The most common form is hereditary non-polyposis CRC, which reportedly accounts for only 2%-5% of CRC cases^[28,29]. Family history, in addition to the genetic syndromes thus far known, constitutes a very significant risk factor for developing CRC, which appears to account for up to 25% of cases^[28]. Although the mechanisms underlying this observation are not completely understood, studies have shown that individuals having first-degree family members diagnosed with CRC are at 2-3 times greater risk of developing CRC than the general population^[28-31].

Although CRC, compared to other common cancers, includes a large percentage of hereditary cases, the vast majority of cases are sporadic, accounting for up to 70%^[28]. The risk factors implicated in the mechanisms of sporadic disease are mainly environmental/acquired. Western lifestyle, cigarette smoking, alcohol intake, obesity and certain dietary habits are amongst the risk factors associated with increased risk for CRC^[24,25,32-34].

PRIMARY PREVENTION

As expected, an important role in the etiology of CRC is attributed to lifestyle factors, since, as aforementioned, the majority of CRC cases are not associated with hereditary/familial factors^[24,25,35]. Western lifestyle is a well-discussed risk factor for CRC, as it was readily observable by researchers that CRC incidence was consistently higher in industrialized countries^[35]. This observation was further supported by the growing incidence in poorer regions as they adopted the western way of living^[24,36].

Diet has been a popular subject of CRC research over the past few decades, both for its potential as a risk factor and as a protective factor. A number of researchers have argued the protective role of a diet high in fiber, with some studies showing a reduction in CRC incidence up to 50%^[35,37-39]. Nevertheless, many recent reports have raised doubts about this argument, leaving the question of how protective dietary fibers really are, open for future prospective studies to answer^[40,41]. Many authors have also asserted a protective role for calcium and vitamin D, as well as for other less verified dietary factors such as folate, vitamin B6, magnesium, garlic and omega 3 fatty acids^[42-46]. On the other hand, frequent consumption of red meat and fat has been associated with increased risk for development of CRC^[47-49].

Obesity has been consistently associated with increased risk for developing CRC, as well as with poorer outcomes following diagnosis^[50-52]. In fact, a review of 29 studies reported that each 5 kg/m² incremental increase in body mass index is accompanied by CRC incidence increase of 24% in men and 9% in women^[50]. In association with a healthy body weight, regular physical activity has been shown to reduce CRC incidence even more, with studies reporting up to 20%-30% lower risk^[53,54].

Alcohol consumption as a risk factor for CRC has been a controversial subject, especially when referring to light and moderate consumption, but studies have consistently reported a higher risk for developing CRC among individuals with moderate to heavy consumption^[55]. Tobacco smoking has been shown to double the risk of being diagnosed with a colon adenoma and to result to poorer outcomes following a cancer diagnosis, leading authors to recommend more intensive screening among smokers^[24,56].

Although no accepted chemopreventive indications exist currently, many pharmaceutical agents have

shown preventive effects against CRC. Aspirin and COX-2 selective inhibitors are among the most investigated agents in regards to CRC prevention, and their regular use has shown ability to reduce incidence in individuals at both average and increased risk^[57,58]. In the general population, the risks from their use seem to outweigh the benefits, but many advocate their use in certain individuals at increased risk for colorectal neoplasia^[59].

SCREENING/SECONDARY PREVENTION

The fact that most CRCs take years to develop - following the Adenoma - Carcinoma sequence - permits the reduction of CRC mortality through screening, either by early detection and removal of the cancer or by detecting and removing the precancerous lesions^[17-19].

There are roughly three categories of screening tests for CRC: stool-based, imaging, and endoscopic tests. Although stool-based tests can reduce mortality rates by early detection of asymptomatic cancerous lesions, imaging and endoscopic tests are capable of further reducing CRC incidence by detecting precancerous lesions as well^[16].

STOOL-BASED TESTS

Guaiac-based fecal occult blood test

Relying on the properties of alpha-guaiaconic acid, a phenolic compound extracted from Guaiacum trees, guaiac-based fecal occult blood test (gFOBT) can detect the presence of heme (of blood hemoglobin) in stool samples. Application of hydrogen peroxide onto guaiac paper causes alpha-guaiaconic acid to oxidize and turn blue. This reaction normally takes time, but heme (if present) catalyzes the reaction and within seconds a blue color change is visible^[60-62]. This bioreactive method was proposed as a screening test for CRC almost half a century ago and has become the most frequently used screen for CRC worldwide^[61].

While the gFOBT is cost affordable and non-invasive, it unfortunately bears many disadvantages. The interpretation of the result is subject to observer bias. Also, the reaction can be catalyzed by any peroxidase, such as heme found in meat, and false-positive results can lead to unnecessary colonoscopies; although, strict dietary restrictions that were proposed in the past seem to now be proven unnecessary^[60,63]. False-negative results, on the other hand, can occur from ingestion of large doses of ascorbic acid (vitamin C)^[64]. Aside from the dietary restrictions related to preparation for the gFOBT, the patient needs to provide three consecutive stool samples in order to achieve adequate sensitivity for occult blood^[65]. The reported sensitivity and specificity vary between studies and different manufacturer brands, and efforts to introduce new, more sensitive

guaiac-based tests resulted in lower specificity^[66]. Finally, this test cannot detect polyps, since they do not bleed, and its sensitivity for advanced adenoma is relatively low^[67].

Fecal immunochemical test

Fecal immunochemical test (FIT) detects blood in stool by using a specific antibody against human hemoglobin. As such, FIT is not affected by diet or observer bias, giving it a greater specificity than gFOBT. Besides specificity, however, its sensitivity for both cancer and adenomas has been shown to be superior to that of gFOBT^[68,69]. According to a recent meta-analysis, the mean reported sensitivity and specificity for FIT detection of CRC is 79% and 94% respectively^[70]. In addition, FIT requires fewer samples than gFOBT, making it more convenient for patients and thus increasing compliance^[71]. Quantitative results can be provided with this method as well, facilitating the ability to determine positive cut-off points for different populations, patient characteristics, or system capabilities and resources^[72].

In summary, stool-based tests are non-invasive and inexpensive methods capable of detecting occult bleeding. However, they are practically incapable of detecting polyps, since the latter do not usually bleed, and they have low sensitivity for detecting adenomas. Consequently, their role in reducing CRC incidence is close to none, but their implementation as a screening tool can reduce CRC mortality by providing early recognition of cancerous lesions. Comparing the two methods, FIT appears superior in terms of sensitivity and specificity (for both CRC and adenomas) and in terms of patient compliance. It is reasonable then to expect that, although more expensive, FIT could be more cost-effective than gFOBT since it could prompt less unnecessary colonoscopies.

IMAGING TESTS

Double-contrast barium enema

In double-contrast barium enema (DCBE), the colon is studied through X-rays obtained after coating the mucosa with barium and distending the colon with air, both of which are inserted transrectal. The DCBE is considered a safe method and has been used frequently in the past, but its use has been dramatically reduced as novel imaging methods become available. The reported sensitivity of DCBE for large polyps (> 10 mm) is only about 50%, and false positive results can occur due to inadequate bowel preparation^[73,74].

Computed tomographic colonography

This method was first described more than 20 years ago, and provides 2- and 3-dimensional endoluminal images of the colon upon reconstructing of computed tomography or magnetic resonance images of the air-

distended colon^[75,76]. The reported diagnostic value of computed tomographic colonography (CTC) has varied between studies, but as newer techniques of CTC are developed it is closing in on colonoscopy in terms of sensitivity and specificity for detecting CRC^[77]. In a recent meta-analysis, the overall sensitivity and specificity of CTC was 66.8% and 80.3% respectively, both lower than the values for colonoscopy. For polyps > 10 mm though, the meta-analysis showed greater sensitivity and specificity (91.2% and 87.3% respectively)^[78].

CTC appears to be more preferred by patients than colonoscopy; in addition, it has a very low risk of bowel perforation and requires no sedation^[79,80]. On the other hand, CTC requires follow-up colonoscopy after positive results (to perform excision/biopsy), exposes the patient to radiation, and the lack of standardized methods leads to variable diagnostic performance^[77,78]. The need for aggressive bowel preparation has been an issue, but newer techniques have been reported involving laxative-free CTC using "fecal tagging" with an ingested contrast agent^[81]. Many authors include in CTC's advantages the potential of discovering extra-colonic pathology in asymptomatic patients, but this argument is controversial since these findings can sometimes lead to unnecessary patient anxiety, costly investigations and overdiagnosis^[82-83].

Colon capsule endoscopy

The colon capsule endoscopy (CCE) method for CRC screening was initially introduced in 2006, and roughly consists of swallowing a pill-shaped device which is capable of photographing the gastrointestinal tract as it passes through it^[84]. Initially, CCE did not gain significant acceptance as a screening tool for CRC, mainly because of its cost and relatively low diagnostic value compared to colonoscopy^[85]. After introduction of the second-generation CCE (CCE-2) in 2009, the subject of CCE has become very popular in the medical literature^[86]. The reported average sensitivity and specificity for the CCE-2 is 86% and 71% respectively, and since 2012 it has been prompted as an acceptable screening method for CRC by the European Society of Gastrointestinal Endoscopy^[87]. Compared to colonoscopy, CCE might be a lot more preferable for the patient, but it is more expensive, lacks excision/biopsy ability and requires very aggressive bowel preparation^[87,88].

ENDOSCOPIC TESTS

Flexible sigmoidoscopy

Flexible sigmoidoscopy (FS) enables the trained physician to visualize the distal gastrointestinal tract up to the splenic flexure, using a flexible, 60 cm long endoscope^[89]. FS requires only minimal bowel preparation, no diet restrictions and no sedation, and can be performed by non-gastroenterologists

(e.g., PCPs) or even trained nurses^[89-91]. Obviously, FS is unable to detect lesions in the proximal colon, which makes it lacking in sensitivity compared to colonoscopy^[90]. In a meta-analysis, FS appeared to reduce CRC incidence and mortality among screened patients, by 32% and 50% respectively^[92].

Colonoscopy

The traditional method of colonoscopy provides visualization of the entire large bowel and the distal part of the small bowel by using a flexible, 120-cm to 160-cm long endoscope^[93]. It is considered by most the 'gold standard' in CRC screening, mainly because of its high sensitivity and specificity for detecting cancerous and precancerous lesions. It also provides the ability to excise or biopsy detected lesions during the same procedure^[6,13,77,90]. Unfortunately, it is also an expensive method and not free of risk; it also requires sedation and extensive bowel preparation. The reported rate of major complications, such as bleeding or bowel perforation, is approximately 0.1%-0.2%, but could become significantly higher when excisions or biopsies are performed and in elderly or comorbid patients^[93].

PREVENTION: SCREENING

IMPLEMENTATION AND THE ROLE OF PCPS

It is beyond doubt that the burden of CRC can be significantly reduced through primary and secondary prevention. Scientific research over the past few decades has offered, as aforementioned, a variety of options for CRC screening and a better understanding of risk and protective factors for the development of CRC. Unfortunately, underutilization of screening and a lack in preventive policies are being reported^[94-98].

Some European countries still have not implemented national mass screening programs, and others that did have reported low participation rates^[97,98]. In the United States, there has been a significant decrease in incidence and mortality following widespread implementation of screening, but the overall use of screening is still below national standards^[94]. Additionally, it has been reported that uninsured people in the United States and people of low socioeconomic or educational status show much lower participation rates^[99].

Many researchers have attempted to identify the causes of CRC screening underutilization and ways to enhance it. While the barriers and sites of potential improvement have been identified at the levels of the health care system and the patient, most of the authors have advocated for the key role of PCPs.

Patients, in many studies, have shown low awareness concerning CRC screening and its importance. In one particularly insightful study, by Aubin-Auger *et al.*^[97], some patients showed low interest in CRC

screening, while others expressed the belief that CRC screening concerns only high-risk individuals or individuals that do not follow a healthy lifestyle. In a systematic review by Holden *et al.*^[100], the authors found that most patients not being screened state their reason as “not thinking about it”. Strongly indicative of how important public awareness is for CRC screening participation was the “Presidential Effect”, a term given to describe the increase in CRC screening participation of United States’ citizens after the nation’s President, Ronald Reagan, was diagnosed with colon cancer in 1985^[101]. Similar reports of the impact of public figure announcements on cancer have been made, but data show that information given occasionally and in an unorganized manner often leads to short term results and mis- or overutilization of screening services^[102,103].

At the level of health care systems, barriers and possibilities for enhancing CRC screening participation are more obvious. Several studies have revealed the efficacy of organized mass screening programs, especially when using patient reminders^[100,104-107]. Participation rate has also appeared to be further enhanced when PCPs are involved in the invitation process^[104,105]. Findings from studies have also led to authors advocating in favor of informational campaigns that increase public awareness of screening^[108]. Another interesting finding in the literature is that patients with “usual source of care” are more likely to be screened for CRC^[100]. This further highlights the importance of PCPs and family physicians in modern health care systems.

Research has also indicated barriers at the level of PCPs, with troubling findings in some cases. Screening recommendation rates by PCPs seem to remain low^[100,109]. Several PCPs in surveys have reported a lack of knowledge and training, and some have even reported not finding screening to be effective^[97,98,109,110]. The major role of PCPs in the effort to decrease CRC incidence and enhance screening participation has become more than obvious. PCPs constitute the first level of contact in a national health system for individual patients and their families. Their role in preventive medicine, through interventions in lifestyle habits, can effectively reduce CRC incidence, as well as that for other diseases. The unique patient-physician relationship in primary health care, in terms of trust and continuity of care, can effectively contribute to patient compliance, as clearly demonstrated throughout the literature.

Interventions through health care system organization, education of PCPs on CRC screening, prevention and counseling techniques, and in public awareness will, therefore, drastically decrease the burden of CRC^[111].

CONCLUSION

Despite the significant improvements in screening techniques and our understanding of risk and protective

factors, CRC remains a major global health burden. PCPs face a unique challenge in their capabilities and efforts to alter this phenomenon; their role in implementing screening and preventive policies is key to reducing the burden of CRC.

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Behavioral gastroenterology: An emerging system and new frontier of action

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Abstract

Behavioral gastroenterology is a new interdisciplinary science that explores the influence of unhealthy lifestyles and psychological factors on the digestive system and addresses the prevention, diagnosis, treatment, and rehabilitation of digestive diseases. Moreover, the concept of whole-course intervention with a focus on disease prevention and a new model of integrated therapy based on alterations of lifestyle and psychology are being gradually established. This paradigm may substantively impact the prevention and treatment of digestive diseases.

Key words: Behavioral gastroenterology; Lifestyle; Psychological factors

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Core tip: As a new interdisciplinary subject, behavioral gastroenterology shows a new concept of whole-course intervention with a focus on disease prevention and a new pattern of integrated therapy based on lifestyle and psychosocial adjustments. It will help both clinicians and patients to alter the old idea of focusing on traditional drugs or surgery, and this new paradigm is expected to become gastroenterology's new frontier of action.

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INTRODUCTION

Behavioral medicine, a phrase first coined at the Yale meeting in 1977, has been developing rapidly. In 2003, the new discipline of behavioral cardiology took a broad view, concluding that heart disease is not inevitable but rather may develop largely from psychosocial stress and unhealthy lifestyles such as smoking, overeating, and physical inactivity^[1]. Likewise, the effects of psychological-behavioral factors and unhealthy lifestyles on digestive diseases are no less impactful than those of traditional risk factors. Thus, by combining behavioral medicine and gastroenterology, a new pattern of psychological-behavioral treatment for gastroenterological patients has gradually emerged.

Based on the research that has integrated both traditional Chinese medicine and modern medicine, we established a behavioral gastroenterological group in the behavioral-psychosomatic medicine branch of the Guangdong Medical Association in 2012. The new concept of behavioral gastroenterology was first proposed in 2013. As an emerging field of clinical practice and an important part of behavioral medicine, behavioral gastroenterology comprehensively explores unhealthy lifestyles and behavioral-psychological factors that can affect the digestive system. This new discipline also addresses the prevention, diagnosis, and treatment of digestive diseases and rehabilitation of patients. Furthermore, a new concept of whole-course intervention with a focus on disease prevention and a new pattern of integrated therapy based on lifestyle/psychosocial adjustments have gradually been established. It is believed that both lifestyle and psychosocial factors can be improved by behavior modification, during which the patient and the practitioner work together.

BEHAVIORAL FACTORS AND DIGESTIVE DISEASES

Traditional Chinese medicine attaches great importance to lifestyle such as diet and daily routine, which are closely related to health and longevity. Similarly, modern medical research has also demonstrated that the onset of many digestive diseases is closely related to deviations in diet, sleep habits, work shifts, and poor lifestyle choices such as excessive alcohol consumption and tobacco use.

Dietary behavior

Certain types of foods are closely associated with symptoms of gastrointestinal diseases. For example, coffee, black pepper, chocolate, and onions often cause epigastric burning and heartburn, milk and dairy products, beans, bananas, and carbonated drinks often produce gas, and fresh meat, fried foods, wheat, manufactured goods, cakes, sweets, chocolate, citrus

fruits, beans, and onions often can cause early satiety. Wheat (93.33%), milk and manufactured goods containing milk products (83.33%), turnip (71.67%), bacon (55%), banana (53.33%), and onion (46.67%) often lead to intestinal smooth-muscle dysfunction, which constitutes a type of functional dyspepsia (FD)^[2]. Additionally, food consumption plays a key role in irritable bowel syndrome (IBS): more than 60% of IBS patients report the onset or worsening of symptoms after meals - within 15 min for 28% of patients, and within 3 h for 93% of patients^[3]. Most IBS patients (84%) report meal-related symptoms as a reaction to at least one specific food, such as milk and dairy products, beans, or apples^[4,5].

Eating behavior is also associated with digestive diseases. Gastroesophageal reflux disease (GERD) correlates with eating greasy or spicy food or drinking carbonated beverages^[6]. Patients with nonalcoholic fatty liver disease often consume high-energy foods and carbohydrates but ingest fewer foods rich in polyunsaturated fatty acids, vitamins, and minerals^[7]. Consumption of certain foods is closely correlated with digestive diseases. Long-term intake of hot foods and beverages positively correlates with the incidence of esophageal cancer^[8]. Fast intake (12 min or less) has been reported by about 40% of FD patients but in only 17% of healthy people. Compared with nonspecific FD, significant differences in the frequency of meal skipping and fast intake have been reported by patients with dysmotility-like FD and ulcer-like FD^[2]. A large-sample multicenter study in China found that refractory FD patients often have unhealthy eating habits such as skipping meals, eating extra meals, or a preference for sweet food and gas-producing food^[9].

Exercise behavior

A sedentary lifestyle is a risk factor for constipation and nonalcoholic fatty liver disease^[10-11]. Tuteja *et al.*^[12] reported that exercise does not affect symptoms such as constipation but can improve patients' quality of life. A meta-analysis revealed that physical exercise can reduce the risk of the occurrence of esophageal cancer, especially adenocarcinoma^[13].

Sleep behavior

GERD patients often report poor sleep quality^[14]. Fass *et al.*^[15] reported that 50.2% of 505 IBS patients suffer sleep disorders such as easy waking during nighttime sleep or fatigue in the morning. Their study also found that poor sleep quality decreases the threshold of visceral pain and that sleep disorders can induce and aggravate the symptoms of IBS. Sleep disorders have also been reported to be associated with the severity of symptoms and the comorbidity of anxiety for FD patients^[16].

Work behavior

A study found that working a rotating shift can

significantly increase the risk of gastrointestinal disorders and peptic ulcers^[17]. Nojkov *et al.*^[18] reported that the incidence of IBS is 48% for nurses with rotating shifts and 31% for nurses with strictly daytime work, and the probability of functional abdominal pain was 81% for nurses with rotating shifts, 54% for those with day shifts, and 61% for those with night shifts. Sleep quality is closely associated with IBS and the incidence of abdominal pain, which might be related to disruption of the circadian rhythm caused by rotating shifts.

Smoking and drinking habits

A large population-based study from developing countries indicated that smoking is not associated with GERD or epidemiologic FD; however, smoking is significantly associated with clinical FD, postprandial fullness, and epigastric pain^[19]. Lunney PC *et al.*^[20] found that the condition of patients with Crohn's disease can be worsened by smoking, and those investigators recommended that such patients quit smoking. In addition, long-term consumption of alcoholic beverages can induce lesions of the liver or pancreas, and alcohol directly damages the mucosa of the esophagus and stomach, modifies the sphincter pressure, impairs gastrointestinal mobility, and alters gastric acid output^[21].

PSYCHOLOGICAL FACTORS AND DIGESTIVE DISEASES

Psychological factors have received attention with respect to the occurrence and development of digestive diseases. A variety of psychological anomalies can be found in FD or IBS patients, especially those involving symptoms of anxiety and depression^[22]. A series of refractory functional gastrointestinal disease studies by our groups in recent years revealed that the incidence of anxiety and depression are 61.5% and 63.3%, respectively, for refractory FD patients, and the severity of anxiety and depression is greater than for non-refractory FD patients^[23]. Moreover, of 1057 patients, the incidence of anxiety and depression for FD patients with weight loss was 56.04% and 59.90%, respectively, and a higher incidence of anxiety and depression was found for patients of group A (lost $\geq 5\%$ of initial body weight) than for those of group B (lost $< 5\%$ of initial body weight)^[24]. Cruz Ruíz *et al.*^[25] reported that 58% of 100 IBS patients suffered anxiety, whereas 62% of those patients suffered depression, and female patients accounted for more than half. The incidence of symptoms of depression and anxiety was 66.2% and 65.5%, respectively, for the refractory IBS group, which was greater than that for the non-refractory IBS and control groups^[26]. Similarly, a significantly higher percentage of refractory globus patients have anxiety and depression compared with non-refractory globus patients. And the prevalence of moderate-to-severe

anxiety and depression was higher for the refractory globus group than for the non-refractory globus group^[27].

A study from Zhou *et al.*^[28] revealed that functional constipation patients have higher scores for depression and anxiety, and the anorectal squeezing pressure is negatively correlated with these scores; these results indicate that depression and anxiety may contribute to functional constipation. GERD patients are more likely to have emotions such as anxiety, depression, and pessimism than the general population, and such psychological disorders may make people vulnerable to GERD^[29].

BEHAVIORAL AND PSYCHOLOGICAL INTERVENTION

Behavioral gastroenterology advocates a new pattern of integrated therapy involving the adjustment of unhealthy lifestyles and psychological problems. This strategy is a shift from the former paradigm, which ascribed greater importance to the treatment of severe digestive diseases and complications. Furthermore, a series of effective behavioral and psychological interventions have been gradually established.

Dietary behavior intervention

Intermittent fasting is an internationally popular diet method that is effective for the control of body weight and the improvement of metabolic syndrome. Intermittent fasting generally entails normal intake for 5 d a week, but intake of a quarter of the normal calories for the other 2 d (usually 500 calories for females, 600 calories for males). A multicenter and large sample research demonstrated that patient body weight was reduced by 4.3 kg on average after 6 wk of intermittent fasting^[30]. Halberg *et al.*^[31] found that healthy people who underwent 2 wk of intermittent fasting exhibited no obvious body weight loss, but they did show improved insulin sensitivity - that is, the capacity for glucose storage and fat decomposition was increased. This result may be the key to improving metabolic syndrome.

A diet low in fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) (Table 1) has been used recently for the management of functional gastrointestinal symptoms for IBS patients. Eswaran *et al.*^[32] showed that the low FODMAP diet led to a significant improvement in IBS symptoms, particularly pain and bloating. Staudacher *et al.*^[33] reported that more patients in the low FODMAP group expressed satisfaction with their symptom response (76%) compared with the standard-diet group (54%).

Intake control has received increasing attention, and intake reduction has been adopted by the majority of people. The strategy of "eating until you are 60% full", which was reported by the Healthy Aging Research Center of the University of London, was

Table 1 FODMAP food list

Food category	Low FODMAP food (things good to intake)	High FODMAP food (things to avoid/reduce)
Vegetables and Legumes	Alfalfa, Bean sprouts, Brussel sprouts, Butternut squash, Callaloo, Carrots, Celery, Chick peas, Chilli, Chives, Cho cho, Choy sum, Collard greens, Corn, Cucumber, Fennel, Green beans, Green pepper, Ginger, Leek leaves, Lentils	Garlic, Onions, Artichoke, Asparagus, Baked beans, Beetroot, Black beans, Cassava, Cauliflower, Falafel, Leek bulb, Mange Tout, Mixed vegetables, Mushrooms, Peas, sugar snap, Savoy Cabbage, Shallots, Taro
Fruit	Ackee, Bananas, Bilberries, Blueberries, Breadfruit, Cranberry, Clementine, Dragon fruit, Grapes, Kiwifruit, Lime, Mandarin, Orange, Passion fruit, Paw paw, Papaya, Pineapple, Plantain, peeled, Raspberry, Strawberry, Tamarind, Tangelo	Apples, Apricots, Avocado, Blackberries, Blackcurrants, Cherries, Currants, Dates, Goji berries, Grapefruit, Lychee, Mango, Peaches, Pears, Pineapple, Plums, Prunes, Sultanas, Tamarillo, Watermelon
Meats, Poultry, and Meat substitutes	Beef, Chicken, Kangaroo, Lamb, Pork, Prosciutto, Quorn, mince, Turkey, Cold cuts/deli meat/cold meats	Chorizo, Sausages, Processed meat - check ingredients
Fish and seafood	Canned tuna, Fresh fish (e.g., Cod, Haddock, Plaice, Salmon, Trout, Tuna), Seafood (e.g., Crab, Lobster, Mussels, Oysters, Prawns, Shrimp)	—
Cereals, Grains, Breads, Biscuits, Pasta, Nuts, and Cakes	Wheat free breads, Gluten free breads, Almonds, Biscuit, savoury, Biscuit, shortbread, Brazil nuts, Bulgur, Buckwheat, Brown rice, Chestnuts, Cornflour, Polenta, Popcorn, Potato flour, Pretzels, Quinoa, Rice, Sorghum, Walnuts	Wheat containing products, Almond meal, Amaranth flour, Barley including flour, Bran cereals, Bread, Cashews, Cous cous, Einkorn flour, Freekeh, Gnocchi, Muesli bar, Pistachios, Rye, Rye crispbread, Semolina, Spelt flour
Condiments, Dips, Sweets, Sweeteners, and Spreads	Aspartame, Acesulfame K, Barbecue sauce, Capers in vinegar, Capers, salted, Chocolate, Garlic infused oil, Golden syrup, Glucose, Marmalade, Marmite, Soy sauce, Stevia, Sweet and sour sauce, Sucralose, Sugar, Vegemite, Vinegars	Agave, Caviar dip, Fructose, Fruit bar, Hummus, Honey, Jam mixed berries/strawberry, Pesto sauce, Quince paste, Relish /vegetable pickle, Stock cubes, Sugar free sweets containing polyols, Sweeteners, Tahini paste, Tzatziki dip
Drinks and protein Powders	Drinking chocolate powder, Espresso, regular, black, Fruit juice (125 mL and safe fruits only), Malted chocolate powder, Tea (Chai tea weak, Green tea, Peppermint tea, White tea), Water	Beer, Coconut water, Cordial, Fruit juices made of apple, pear, mango and Orange, Rum, Sodas containing High Fructose Corn Syrup (HFCS), Soy milk, Sports drinks, Wine
Dairy foods	Butter, Dairy free chocolate pudding, Eggs, Margarine, Soy protein, Tempeh, Tofu, Whipped cream, Yoghurt with lactose free	Buttermilk, Cheese, Cream, Custard, Gelato, Ice cream, Kefir, Milk, Sour cream, Yoghurt

observed to extend the average lifespan of mice for five or six months - that is, it prolonged life by 30%. This would correspond to 20 years in humans.

Exercise behavior intervention

De Schryver *et al.*^[34] found that regular physical activity (including 30 min of brisk walking and 11 min a day family project) could significantly improve defecation problems (e.g., overly frequent defecation, laborious defecation, and hard stools) caused by a lack of activity in middle-aged patients with chronic idiopathic constipation and significantly shorten the transit time in the sigmoid colon and rectum colon.

In addition, the results from Matsuzaki *et al.*^[35] revealed that the intensity of regular exercise is independently associated with gastric emptying in healthy individuals, and gastric emptying was significantly faster in the low-intensity exercise group than in the moderate-intensity exercise group. It is trusted to be an optimal exercise intervention for the treatment of FD.

Life behavior intervention

Nowak *et al.*^[36] implemented lifestyle adjustments for 23 patients with GERD during one month such as not leaning in during the 2 to 3 h after a meal, having more meals a day but less food at each meal, and a low-fat diet. The frequency and severity of GERD symptoms significantly decreased for 22 patients, and 11 patients reduced their use of GERD-specific drugs.

A report from Eherer *et al.*^[37] revealed that

abdominal breathing training combined with proton pump inhibitors was more effective than proton pump inhibitors alone for the treatment of GERD. Thus, non-drug lifestyle intervention therapy can effectively improve symptoms and reduce the medication burden of patients.

Psychological intervention

Psychological factors are considered to be closely related to functional digestive diseases such as FD, IBS, GERD, and globus. Therefore, it is very important to quickly determine the causes of the psychological problems and help solve them. At the same time, appropriate drugs that can alleviate emotions should be used.

We performed a series of treatment research on the application of low-dose antidepressants in depressive patients with functional gastrointestinal diseases. Amitriptyline (25 mg/d) was used to treat globus patients. After 4 wk of treatment, the amitriptyline group showed significantly greater improvement in the Glasgow Edinburgh Throat Scale score and sleep quality than the group that received conventional treatment (pantoprazole, 40 mg/d)^[38]. Paroxetine (20 mg/d) was also applied for globus treatment. After 6 wk of treatment, 71.7% of the subjects in the paroxetine group (33/46) exhibited a treatment response, which was significantly greater than those for the amitriptyline group (46.2%, 24/52) and the lansoprazole group (14.0%, 7/50). A more distinct improvement of emotional well-being, quality of life,

and quality of sleep was observed in the paroxetine group than in the lansoprazole or amitriptyline group^[39]. Additionally, mirtazapine (30 mg/d) was utilized to treat the FD patients with weight loss. After 8 wk of treatment, mirtazapine not only alleviated symptoms associated with dyspepsia and depression linked to FD for patients with weight loss but also significantly increased body weight (mainly the visceral fat in body fat)^[40].

The low-dose administration of antidepressants mentioned above may not only alleviate the symptoms of depression but also improve the patient's digestive symptoms. Because these satisfactory effects can be achieved at low medical cost, the treatments are worth promoting in the clinic.

CONCLUSION

Unhealthy lifestyle and psychological factors are closely related to the occurrence, development, curative effect, and prognosis of digestive diseases. Thus, the new concept of whole-course intervention with a focus on disease prevention, and the new model of integrated therapy for the adjustment of unhealthy lifestyles and psychological problems are being established. As a new interdisciplinary subject, behavioral gastroenterology will help both clinicians and patients to alter the old idea of focusing on traditional drugs or surgery while ignoring lifestyle adjustments, and this new paradigm is expected to become gastroenterology's new frontier of action.

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Basic Study

Pharmacological evaluation of NSAID-induced gastropathy as a "Translatable" model of referred visceral hypersensitivity

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Author contributions: Hummel M and Whiteside GT equally conceptualized study design, analysis, and interpretation of study results; Hummel M wrote the manuscript; Knappenberger T collected and analyzed the data; Reilly M collected rotarod data and prepared the figures depicting the data.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Purdue Institutional Animal Care and Use Committee (IACUC Protocol number: 2014-100) in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Ethical Guidelines of the International Association for the Study of Pain (www.iasp-pain.org) and are reported in accordance with the ARRIVE guidelines (www.nc3rs.org.uk). All efforts were made to minimize the number of animals used and to avoid any undue pain.

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Abstract

AIM

To evaluate whether non-steroidal anti-inflammatory drugs (NSAIDs)-induced gastropathy is a clinically predictive model of referred visceral hypersensitivity.

METHODS

Gastric ulcer pain was induced by the oral administration of indomethacin to male, CD1 mice ($n = 10/\text{group}$) and then assessed by measuring referred abdominal hypersensitivity to tactile application. A diverse range of pharmacological mechanisms contributing to the pain were subsequently investigated. These mechanisms included: transient receptor potential (TRP), sodium and acid-sensing ion channels (ASICs) as well as opioid receptors and guanylate cyclase C (GC-C).

RESULTS

Results showed that two opioids and a GC-C agonist, morphine, asimadoline and linaclotide, respectively, the TRP antagonists, AMG9810 and HC-030031 and the sodium channel blocker, carbamazepine, elicited a dose- and/or time-dependent attenuation of referred visceral hypersensitivity, while the ASIC blocker, amiloride, was ineffective at all doses tested.

CONCLUSION

Together, these findings implicate opioid receptors, GC-C, and sodium and TRP channel activation as

possible mechanisms associated with visceral hypersensitivity. More importantly, these findings also validate NSAID-induced gastropathy as a sensitive and clinically predictive mouse model suitable for assessing novel molecules with potential pain-attenuating properties.

Key words: Visceral hypersensitivity; Pain; Translation; Guanylate cyclase C; Non-steroidal anti-inflammatory drugs; Transient receptor potential channel

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Core tip: Recently, standard animal models of pain have been vehemently challenged for their inability to successfully predict human clinical outcomes. Further, few animal models have been represented with reasonable translational value for conditions presenting with visceral pain. Non-steroidal anti-inflammatory drug -induced gastropathy represents a translatable model of visceral hypersensitivity in which several pain targets have demonstrated reliable sensitivity when assayed. Further, this model is robust enough that proper pharmacological evaluation can be conducted. Overall, this model has the potential to efficiently triage molecules with pain-attenuating properties for their utility in gastrointestinal disorders that include pain as a hallmark symptom.

Hummel M, Knappenberger T, Reilly M, Whiteside GT. Pharmacological evaluation of NSAID-induced gastropathy as a "Translatable" model of referred visceral hypersensitivity. *World J Gastroenterol* 2017; 23(33): 6065-6076 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6065.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6065>

INTRODUCTION

Stomach ulcers are common and afflict millions of individuals annually (www.mayoclinic.com)^[1]. Like many gastrointestinal (GI) disorders, stomach ulcers cause significant pain, a hallmark feature of many ailments affecting visceral organs. Although stomach ulcers predicate a type of visceral hypersensitivity, surprisingly the mechanisms underlying the pain associated with gastric ulceration as with some other GI conditions [*i.e.*, irritable bowel syndrome (IBS), functional dyspepsia, pancreatitis] remain poorly understood^[2-4]. Several preclinical models of gastric or duodenal ulceration have been established that highly resemble human ulcers in terms of pathological features including the indomethacin-induced gastropathy and acetic acid models^[5-9]. While these and other models have been used to aid the development of novel disease ameliorating therapies (*i.e.*, proton pump inhibitors and histamine receptor antagonists) as well as elucidate the notable involvement of

Helicobacter pylori infection in ulcer formation, a direct behavioral measure of the pain associated with ulceration in these models has not been reported^[10]. Furthermore, despite the success of these drugs in healing ulcer lesions, sensory aberrations leading to such pain have not been clearly delineated and often remain a chief complaint for many patients^[10,11]. This is especially true for those individuals who are actively treated for ulcers but who also require concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs) or aspirin, analgesics commonly used for other chronic conditions such as osteoarthritis (OA) and cardiovascular disease^[12,13]. In conditions like OA, these agents are used to treat musculoskeletal pain, but paradoxically cause or exacerbate stomach pain on their own^[13,14]. NSAIDs and salicylates are ulcerogenic and therefore, chronic use can exacerbate existing gastric injury or lead to new ulcer formation^[15]. For these and other patients, it has been hypothesized that persistent or unresolved visceral pain, despite the etiology, may be due to aberrations in primary afferent function or hypersensitivity, peripheral sensitization, and/or psychological/genetic abnormalities^[16-20].

With this in mind, we characterized the pain associated with gastric ulceration. By combining a clinically relevant stomach ulcer model with a predictive behavioral endpoint, we investigated some potential mechanisms producing visceral hypersensitivity. To this end, we used the indomethacin-induced gastropathy model to model the mucosal injury and concomitant pain associated with NSAID use. This model recapitulates the human condition in that indomethacin is orally administered to mice to produce mucosal damage, inflammation and referred visceral hyperalgesia^[21-23]. Like in other GI disorders, ulcer pain is diffuse. Moreover, it can be referred to somatic structures and may present itself atypically given the dichotomization of sensory fibers that innervate visceral tissues^[24-27]. Therefore, since ulcer pain is reportedly present upon palpation or mechanical stimulation of the abdomen both in dogs^[28] and humans^[29], we extrapolated this to mice and quantified the referred abdominal hypersensitivity by measuring the number of behavioral responses evoked by von Frey fiber stimulation^[23,30]. We then investigated the pharmacological role of guanylate cyclase C (GC-C) and opioid receptors as well as TRPs, ASICs and sodium channels in this regard since all have been implicated in visceral hypersensitivity and/or functional bowel disorders to some degree^[31-35].

MATERIALS AND METHODS

Animals

Male CD-1 mice (Harlan Laboratories, Indianapolis, IN, United States) weighing 20-25 g were housed on cob bedding (five/cage) in a climate-controlled room and maintained on a 12-h light/dark cycle with free access

to food and water. Animals were acclimated to the Purdue Pharma L.P. animal facility for one week prior to testing.

Animal care and use statement

All studies were approved by the Purdue Institutional Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Ethical Guidelines of the International Association for the Study of Pain (www.iasp-pain.org) and are reported in accordance with the ARRIVE guidelines (www.nc3rs.org.uk). All efforts were made to minimize the number of animals used and to avoid any undue pain.

Ulcer pain model

As previously described, mice were fasted overnight then dosed orally with 30 mg/kg indomethacin to develop the ulcer model^[23,30]. Control animals received vehicle (10 mL/kg, p.o.). Morphine was administered 2 h post-indomethacin while the other compounds were administered 3 h post-indomethacin or 1 h before testing. Stomachs from a separate set of vehicle- and indomethacin-dosed mice were dissected and photographed to ensure ulcer model development.

Behavior

Referred abdominal hypersensitivity from indomethacin-induced gastric ulceration was quantified by measuring the threshold to withdrawal from the application of a tactile stimulus to the abdominal area^[23]. Briefly, the abdominal area was shaved and the mice were subsequently placed inside Plexiglas boxes situated on elevated wire screen mesh flooring conducive to von Frey probing. Following a habituation period, baseline tactile sensitivity was assessed. Tactile hypersensitivity was then measured 4 and 24 h following indomethacin administration using von Frey filaments applied to the upper abdomen (bending force 0.005–3.58 g starting with the 0.407 g fiber). The upper abdominal region was stimulated with an incremental series of eight monofilaments of increasing logarithmic stiffness. The 50% withdrawal threshold was determined using the up-down method of Dixon, modified by Dixon^[36] and Chaplan *et al.*^[37]. First, an intermediate von Frey monofilament (number 3.58) was applied to the abdomen causing a slight bending. If a positive response was noted (abdominal/whole body withdrawal or licking), a smaller filament was then tested. Conversely, if no response occurred, the next larger filament was tested. If an animal did not respond to monofilament application, a cut-off value of 3.58 was assigned to that mouse^[23,30]. A separate set of naïve mice were also dosed with the highest doses of the compounds of interest. von Frey thresholds were determined 4 h and 24 h post-indomethacin administration, noting that at least four hours is needed before a pain response can be reliably measured.

Rotarod

To delineate efficacy and assess changes in motor performance following compound administration, naïve mice were tested on an automated accelerating rotarod (Ugo Basile, Italy). For training and testing, the rotarod speed was increased from 4 to 40 rpm over a 300 second period. The maximum time spent on the apparatus was set to 300 s. Mice received two training sessions separated by a minimum of 2 h on the first day then 24 h later they were administered compound or vehicle. The latency to fall was assessed 1, 2 and 24 h post-compound administration. It is important to note that only the highest dose tested in the efficacy assessment was evaluated in the rotarod assay.

Blinding and randomization

All testing was conducted in a blinded manner with experimenters involved in the study being unaware of the group assignment of any animal they were testing. Dosing solutions were coded using A–E. Animals were dosed in a blinded fashion after pre-treatment baseline assessment such that animals were assigned to treatment groups based on baseline response thresholds so that group means were approximately equal (*i.e.*, randomization). Briefly, animals were ranked by response threshold from lowest to highest and treatments were assigned as follows (*e.g.*, A, B, C, D, E, B, C, D, E, A, C, D, E, A, B, D, E, A, B, C, *etc.*). The animals were then dosed in sequence based on animal number, so that the distribution of treatment across a given set of animals was not predictable.

Statistical analysis

All data are expressed as the mean \pm SEM. Data were analyzed using a two-way repeated measures analysis of variance (ANOVA) using the factors of treatment and time. The Bonferroni multiple comparisons test was subsequently performed for post-hoc comparison using GraphPad Prism software (version 5.04; GraphPad Software, Inc., United States). Statistical significance was set to $P < 0.05$. The statistical methods of this study were reviewed by Salvatore Colucci, a biomedical statistician employed by Purdue Pharma L.P.

Compounds

Indomethacin, AMG 9810, HC-030031, carbamazepine, amiloride HCl and morphine were obtained from Sigma-Aldrich (St. Louis, MO, United States). Linaclotide, and asimadoline HCl were obtained from Toronto Research Chemicals (Toronto, ON, Canada). AMG 9810, HC-030031, asimadoline and linaclotide were administered p.o. as suspensions in 0.5% methylcellulose. Amiloride HCl was formulated in 25% hydroxypropyl- β -cyclodextran and administered intraperitoneally while morphine was dissolved in saline and given subcutaneously. Also, with the exception of morphine, all compounds were administered one hour prior to behavioral testing (three hours post-

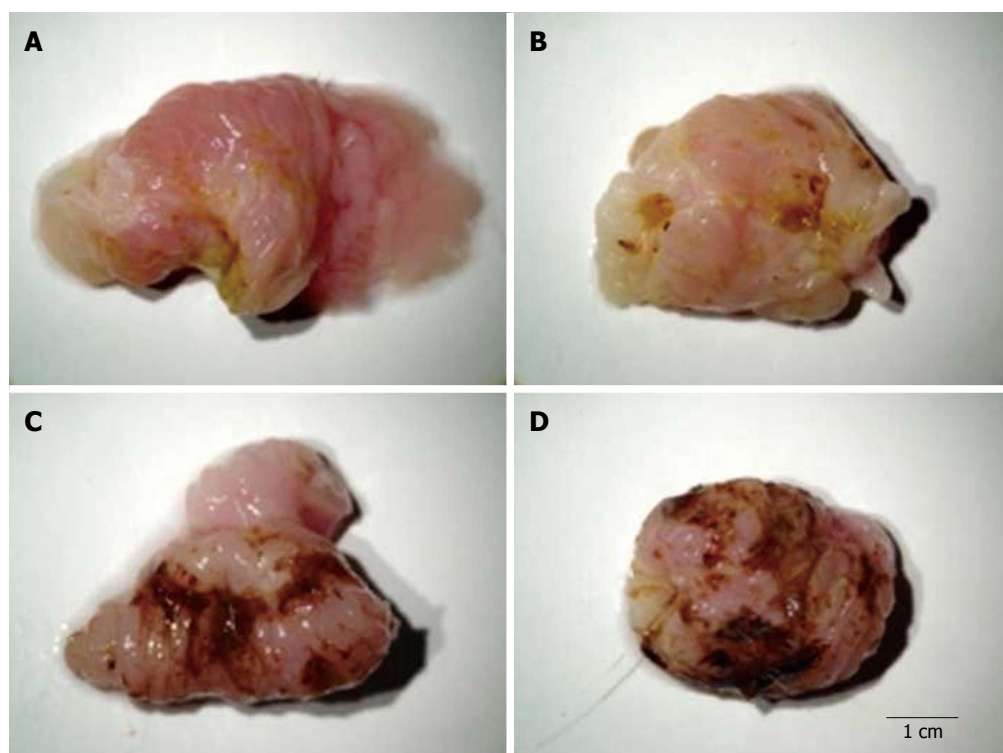


Figure 1 Representative digital photographs of stomachs dissected from mice orally dosed with vehicle (A) or 30 mg/kg indomethacin (B-D). Stomachs were scored as follows: A: score 0 = no lesion (normal mucosa = vehicle); B: score 1 = light petechial; C: score 2 = diffuse bleeding or aphtha; and: score 3 = multiple aphtha.

indomethacin dosing). Morphine was administered two hours before testing (or two hours after indomethacin). Route, vehicle and pre-treatment time for all compounds were chosen based on preliminary dosing experiments, pharmacokinetic (PK) data and/or previously published work. All doses are expressed as the free base and were administered in a dose volume of 10 mL/kg.

RESULTS

Ulcer model

Model development: We removed stomachs from vehicle- and indomethacin-dosed mice (4-h post-indomethacin administration). Mice were humanely euthanized. Their stomachs were grossly dissected, rinsed with saline and then photographed to demonstrate model development as shown in Figure 1. It is important to note that we found that visceral sensitivity is present in indomethacin-dosed animals irrespective of the degree of mucosal injury present. Behaviorally, severe mucosal damage does not always produce the most robust pain response.

Efficacy assessment

Opioid receptors: We examined the effect two distinct opioid receptor agonists had on visceral hypersensitivity. Firstly, we assessed the effect morphine (Figure 2A), the prototypical mu opioid receptor (MOR) agonist, had

on the evoked visceral pain response. Results showed successful ulcer model development both 4 and 24-h post-indomethacin dosing ($P < 0.05$ vs vehicle), and that morphine attenuated the indomethacin-induced visceral hypersensitivity with a minimal effective dose (MED) equal to 10 mg/kg. Although there was a main effect of treatment [$F(4, 54) = 36$, $P < 0.05$ vs indomethacin] and a significant interaction with time [$F(8, 79) = 21$, $P < 0.05$ vs indomethacin], the effect morphine elicited on the pain behavior was only noted 2 h post-dosing (4 h post-indomethacin) for each of the higher doses tested, 10-30 mg/kg. Morphine was not efficacious in the assessment conducted 24-h post-indomethacin dosing. Nonetheless, the short-lived efficacy is consistent with the half-life of this drug. Subsequently, we next examined the effect asimadoline, a selective kappa opioid receptor (KOR) agonist, had in this pain model as well. Results showed consistent model development both 4 and 24-h post-indomethacin dosing ($P < 0.05$ vs vehicle). Asimadoline attenuated the indomethacin-induced visceral hypersensitivity with a MED= 10 mg/kg (Figure 2B). Like morphine, asimadoline was also efficacious in the model (10-30 mg/kg). There was a main effect of treatment [$F(4, 63) = 25$, $P < 0.05$ vs indomethacin] and a significant interaction with time [$F(2, 78) = 52$, $P < 0.05$]. However, unlike morphine, the kappa driven opioidergic effect was more prolonged such that the 30 mg/kg dose of asimadoline continued to attenuate the

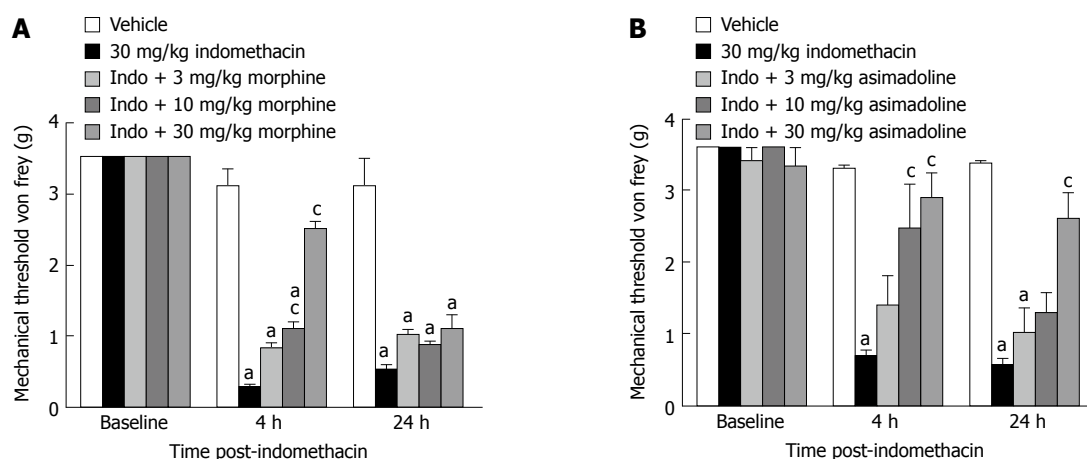


Figure 2 Effect of opioid receptor agonism on gastric ulcer pain. A: Dose-response and time course for the effect of a mu opioid receptor agonist on referred gastric ulcer pain. Morphine significantly attenuated referred gastric ulcer pain when dosed 10-30 mg/kg; B: Dose-response and time course for the effect of a selective kappa opioid receptor agonist on referred gastric ulcer pain. Asimadoline significantly attenuated referred gastric ulcer pain when dosed 10-30 mg/kg. All data are expressed as the mean \pm SEM, where $n = 8-10$ mice/group. ^a $P < 0.05$ vs vehicle, ^c $P < 0.05$ vs indomethacin.

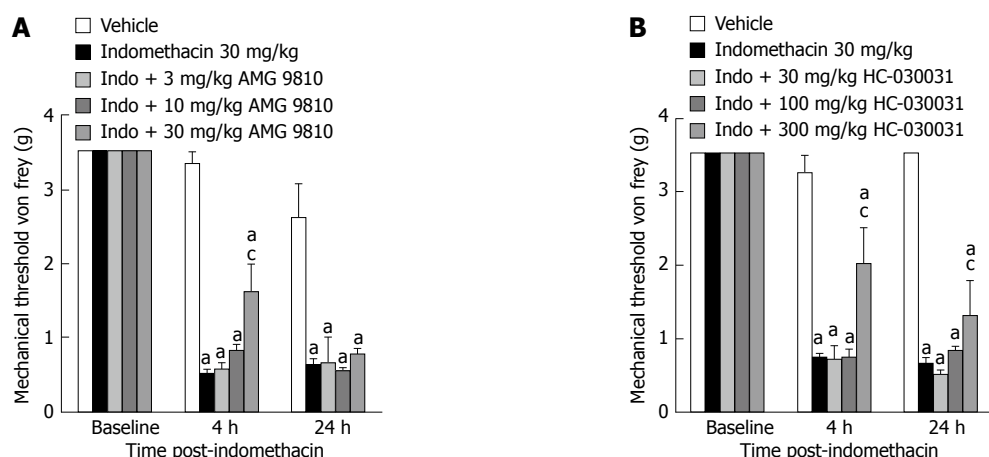


Figure 3 Effect of transient receptor potential channel blockade on gastric ulcer pain. A: Dose-response and time course efficacy for selective TRPV1 channel blockade on referred gastric ulcer pain. AMG 9810 significantly attenuated referred gastric ulcer pain when dosed at 30 mg/kg; B: Dose-response and time course for the effect of selective TRPA1 channel blockade on referred gastric ulcer pain. HC-030031 significantly attenuated referred gastric ulcer pain when dosed at 300 mg/kg. All data are expressed as the mean \pm SEM where $n = 10$ mice/group. ^a $P < 0.05$ vs vehicle, ^c $P < 0.05$ vs indomethacin.

evoked pain response even at the 24-h assessment.

TRP channels

Next, we assessed the effect two distinct TRP channel receptor antagonists had on the visceral hypersensitivity. Firstly, we assessed the effect that AMG 9810, the most studied TRPV1 receptor compound, had on the evoked visceral pain response. In the ulcer pain model, indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 3A, $P < 0.05$ vs vehicle). Systemic treatment with the TRPV1 antagonist AMG 9810 attenuated the referred ulcer pain by increasing the mechanical threshold at which a behavioral response was elicited. Although there was a main effect of treatment [$F(4, 44) = 43.2$, $P < 0.05$ vs indomethacin alone] and a significant interaction with time [$F(8, 75) = 15$, $P < 0.05$], only

the 30 mg/kg dose significantly attenuated the visceral hypersensitivity present 4 h post-dosing as revealed by post hoc comparison. Efficacy was not observed at the 24-h assessment.

We then examined the effect HC-030031, a selective transient receptor potential ankyrin 1 receptor (TRPA1) antagonist, had in this pain model as well. Indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 3B, $P < 0.05$ vs vehicle). Similar to TRPV1 blockade, the systemic treatment with the TRPA1 antagonist HC-030031 also significantly attenuated the referred ulcer pain in indomethacin dosed mice (Figure 3B). Although there was a main effect of treatment [$F(4, 46) = 38$, $P < 0.05$ vs indomethacin alone] and a significant interaction with time [$F(8, 71) = 15.4$, $P < 0.05$], only the 300 mg/kg dose attenuated the visceral hypersensitivity as noted

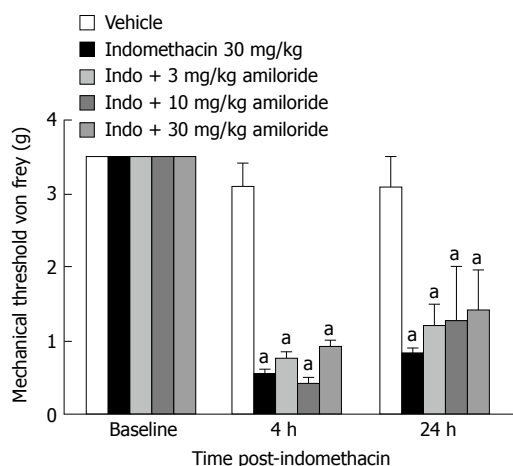


Figure 4 Effect of acid-sensing ion channels channel blockade on gastric ulcer pain. Dose-response and time course for the effect of non-selective ASIC channel blockade on referred gastric ulcer pain. Amiloride did not attenuate referred gastric ulcer pain at any of the doses tested. Data are expressed as the mean SEM where $n = 10$ mice/group. $^aP < 0.05$ vs vehicle.

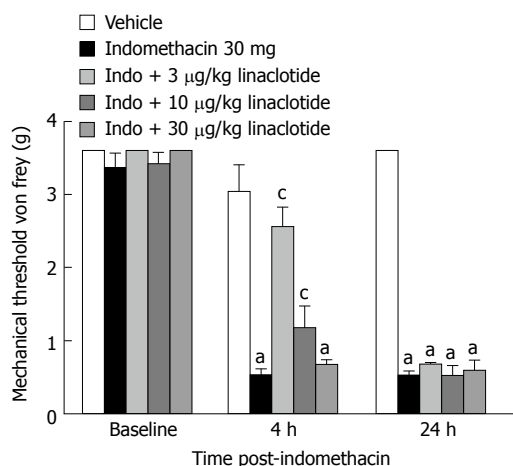


Figure 5 Effect of guanylate cyclase C agonism on gastric ulcer pain. A: Dose-response and time course for the effect of linacotide on referred gastric ulcer pain. Linacotide significantly attenuated referred gastric ulcer pain when dosed 3-10 µg/kg. Data are expressed as the mean SEM where $n = 9-10$ mice/group. $^aP < 0.05$ vs vehicle, $^cP < 0.05$ vs indomethacin.

by post-hoc comparison. Unlike the TRPV1 antagonist AMG9810, the effect of 300 mg/kg HC-030031 persisted at the 24-h assessment.

ASICs: We also investigated the sensory role of ASICs in ulcer pain. In the ulcer pain model, indomethacin-dosed mice demonstrated reduced mechanical thresholds to von Frey fiber application compared to vehicle-treated animals as previously shown both 4 and 24-h post-dosing (Figure 4; $P < 0.05$). Surprisingly, however, the non-selective ASIC blocker amiloride did not significantly reduce the referred ulcer pain at any doses tested when evaluated by post-hoc comparison. Referred abdominal hypersensitivity was present in all dose groups 4 and 24-h post-dosing.

Guanylate cyclase-C: We determined if the newly developed GC-C agonist, linacotide, was efficacious at attenuating ulcer pain. Indomethacin dosed mice demonstrated reduced mechanical thresholds compared to vehicle-dosed animals both 4 and 24-h post-dosing (Figure 5; $P < 0.05$). There was a main effect of indomethacin treatment [$F(4, 62) = 486$, $P < 0.05$] and a significant interaction with time, [$F(2, 128) = 64$; $P < 0.05$] 4 h post-dosing. 3-10 µg/kg linacotide (MED = 3 µg/kg) significantly attenuated the indomethacin-induced mechanical hypersensitivity ($P < 0.05$). Surprisingly, the 3 µg/kg dose produced the most efficacious response while the 30 µg/kg dose was without effect. Visceral hypersensitivity was still notably present in mice that received 30 µg/kg linacotide. That is, their tactile response was consistent with vehicle-dosed controls. The three doses of linacotide tested were not efficacious in the ulcer pain model during the 24-h assessment.

Sodium channels: Lastly, we determined if the non-selective sodium channel blocker, carbamazepine, was efficacious in the ulcer model. Indomethacin dosed mice demonstrated reduced mechanical thresholds compared to vehicle-treated animals both 4 and 24-h post-dosing (Figure 6, $P < 0.05$). There was a main effect of indomethacin treatment ($F(4, 58) = 14$, $P < 0.05$) and a significant interaction with time ($F(2, 71) = 65$, $P < 0.05$). The 100 mg/kg dose of carbamazepine significantly reversed the referred tactile hypersensitivity noted during the 4-h assessment ($P < 0.05$). Those animals that received the 100 mg/kg dose of carbamazepine also demonstrated significantly less visceral hypersensitivity during the 24-h assessment as well ($P < 0.05$ vs indomethacin).

Side effect assessment

von Frey Threshold: To determine whether the compounds used in these experiments had any activity in the absence of visceral hypersensitivity, we assessed whether the highest doses tested in the efficacy model affected the von Frey thresholds of naïve animals. Post hoc analysis revealed that amiloride (Figure 7A; $P < 0.05$ vs vehicle), linacotide, asimadoline and carbamazepine (Figure 7B; $P < 0.05$ vs vehicle) altered the von Frey thresholds of naïve mice when dosed at the highest dose examined in the ulcer model. Tactile sensitivity was enhanced 4-h after the dosing of linacotide, asimadoline and carbamazepine, and 4 and 24-h after the administration of amiloride.

Rotarod performance: To place the efficacy of the compounds assessed into context with any inherent side effect liability, the highest dose of each compound examined in the ulcer model was subsequently evaluated in naïve mice using the rotarod assay. Morphine produced a slight but non-significant decrease

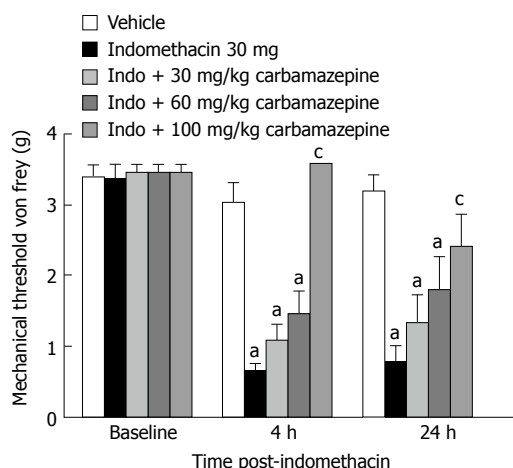


Figure 6 Effect of sodium channel blockade on gastric ulcer pain. Dose-response and time course for the effect of carbamazepine on referred gastric ulcer pain. Carbamazepine significantly attenuate referred gastric ulcer pain when dosed at 100 mg/kg. Data are expressed as the mean \pm SEM with 9-10 mice/group. ^a $P < 0.05$ vs vehicle, ^c $P < 0.05$ vs indomethacin.

in rotarod performance one hour after administration (Figure 8A), while carbamazepine produced significant deficits in rotarod performance both 1 and 2 h following dosing (Figure 8B; $P < 0.05$ vs vehicle). Conversely, AMG 9810, HC-030031, linaclotide, asimadoline and amiloride did not produce any deficits in motor performance as show.

DISCUSSION

Pain is a characteristic feature of many chronic disorders affecting the GI tract. In this study we measured the pain associated with NSAID-induced gastropathy^[13,14] and then investigated relevant pharmacological mechanisms underlying its development and maintenance.

Firstly, our findings show that the NSAID-induced gastropathy model in mice consistently produces stomach ulceration and referred abdominal hypersensitivity that can be reliably measured. Importantly, the model is robust enough that proper pharmacological evaluation can be conducted. Since gastric ulcers are a source of visceral pain which can be referred to somatic dermatomes upon palpation of the abdomen^[28,29], this study was able to measure the threshold at which mice responded to abdominal application of von Frey filaments. Although this response was used as an index of the pain, it was no surprise that the mice demonstrated such robust behavior given the macroscopic changes depicted in Figure 1.

In characterizing the mechanisms associated with this pain model, we first assessed the effects of two distinct opioid analgesics. We determined that the MOR agonist, morphine, attenuated the ulcer pain in a dose- and time-dependent manner. This was not unexpected given the robust presence of MORs on enteric neu-

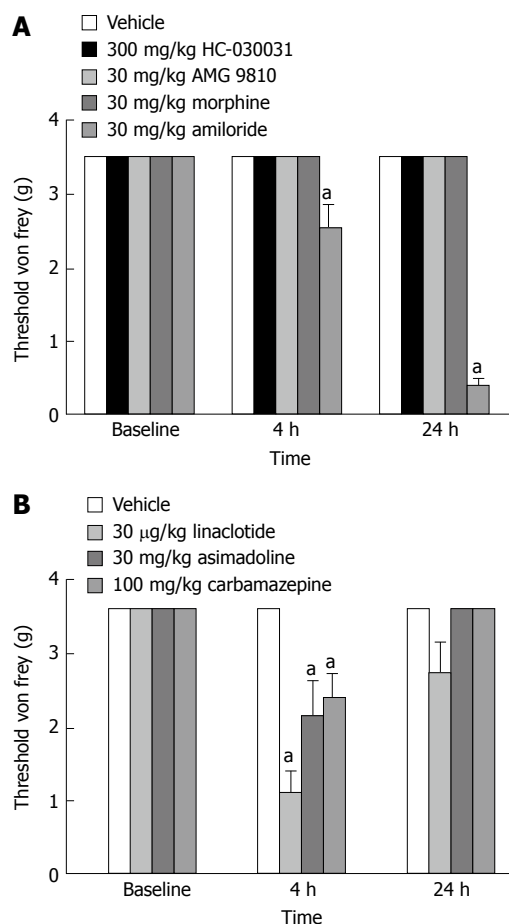


Figure 7 Effect of compound administration on von Frey threshold in naïve mice. A: The non-selective ASIC blocker amiloride significantly decreased abdominal threshold in mice 4 and 24-h post-administration when dosed at 30 mg/kg. B: Linaclotide, asimadoline and carbamazepine significantly decreased abdominal threshold in mice 4-h post-administration. Data are expressed as the mean \pm SEM, where $n = 9-10$ mice/group. ^a $P < 0.05$ vs

rons of the GI tract, as well as this compound's well-published *in vitro* and *in vivo* pharmacological profile^[38]. Interestingly and unknowing to us at the time of dosing was that the effect morphine had on ulcer pain may extend beyond its historical pain-ameliorating actions at the receptor level. That is, morphine may also provide gastric defense. An early study reported that morphine is protective to the stomach because it increases mucous production and decreases acid secretion^[39]. However, contrary to this effect, it is also noteworthy to mention that morphine may be pro-ulcerogenic in certain circumstances as well. Esplugues and Whittle^[40] had suggested that morphine can potentiate acid- and ethanol-induced gastric injury, although the experimental conditions employed herein did not support this result. Nonetheless, in this investigation, we demonstrated that morphine attenuated referred visceral pain at doses that did not alter von Frey thresholds or produce ataxia in naïve mice. However, it is important to note that the utility of MOR agonists for the treatment of visceral pain needs to be judiciously scrutinized given their effect on GI

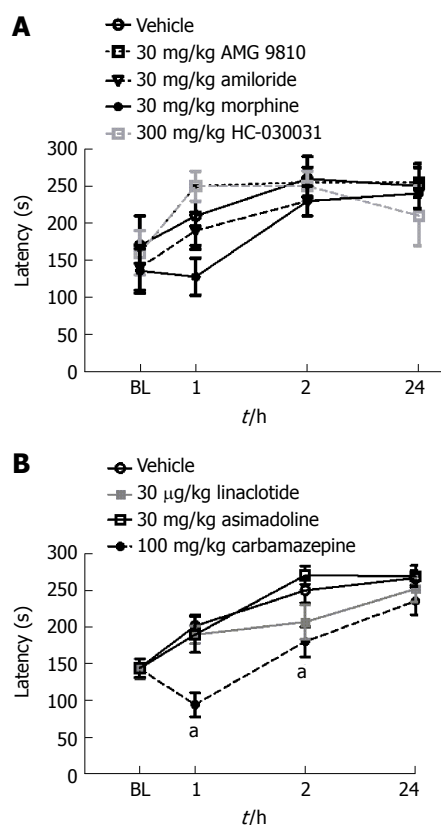


Figure 8 Effect of compound administration on rotarod performance. A: AMG 9810, amiloride, morphine and HC-030031 did not produce any significant deficits in rotarod performance 1-24 h post-administration; B: Linaclootide and asimadoline were without effect while carbamazepine significantly altered rotarod performance 1 and 2-h post-administration, ^a $P < 0.05$. All data are expressed as the mean \pm SEM, where $n = 10$ mice/group.

motility.

We next examined the efficacy of asimadoline, a potent KOR agonist. Asimadoline has 500-fold selectivity for kappa versus MORs and it is purported to produce its analgesic actions peripherally rather than centrally^[41,42]. Previous work in animals has suggested that KOR agonists may represent a more viable approach to treating painful visceral conditions due to the nature of KOR expression and function^[43,44]. In this regard, we hypothesized that asimadoline may be beneficial in attenuating visceral hypersensitivity. Interestingly, on a dose-per-dose basis, asimadoline was more efficacious than morphine in the model. Like morphine, it did not produce any deficits in rotarod performance at the highest dose tested; however, the 30 mg/kg dose did enhance tactile sensitivity in naïve animals. While Delgado-Aros *et al.*^[45] found that asimadoline produced hyperalgesia in patients dosed with high doses of the compound, there is a disconnect between human and rodent. In the human condition, these results may suggest that the KOR system becomes engaged during pathological conditions of persistent hypersensitivity^[41,46] and that it does not significantly alter noxious sensation in healthy states^[44]; however, these findings/assumptions

are not consistent with ours since the mice tested were naïve and not subjected to ulcer induction. Although continued research is necessary to further our understanding on how KOR agonists modulate visceral pain, there is clinical evidence to support such an indication. Most notably, Cara Therapeutics have recently developed a peripherally restricted compound, CR665, which has demonstrated efficacy in human visceral pain^[47-48].

TRP channels have received a significant amount of attention for their alleged role in pain including visceral hypersensitivity^[35,49]. Since TRP channels transduce noxious stimuli at the peripheral terminals of nociceptors, these channels are well-poised anatomically to function as the first integrators of the pain response^[50]. In support of this Akbar and colleagues^[51] demonstrated a 3.5-fold increase in the density of TRPV1 immuno-reactive fibres in the colonic biopsies of patients with IBS compared to healthy controls. Transient receptor potential vanilloid-1 (TRPV1) is a ligand-gated nonselective polymodal cation channel that integrates a variety of pain stimuli^[49]. Since noxious GI events are conveyed to the CNS by vagal and spinal afferents, and TRPV1 is expressed within both dorsal root and nodose ganglia innervating the GI tract^[52,53], we examined whether selective blockade of the TRPV1 receptor would attenuate ulcer pain. Our results showing the ability of AMG 9810 to attenuate pain in the ulcer model suggests that the irritating effects of indomethacin on the gastric mucosa may engage the TRPV1 receptor either directly or indirectly such that these afferent neurons become hypersensitive to the hydrochloric acid that is normally innocuous to the stomach. Overall, the net effect may be analogous to proton activation of the channel. Alternately, it also becomes noteworthy to consider early reports supporting the protective effects of capsaicin-sensitive afferent neurons on the gastric mucosa^[16,54]. While activation is believed to be protective, ablation of capsaicin-sensitive neurons impairs mucosal defense. With regard to our findings, one may have expected antagonism of TRPV1 to be detrimental. However, unlike nerve ablation which removes the entire nerve terminal and its functionality, an antagonist would leave the protective effects of these neurons intact.

Like TRPV1, TRPA1 is a non-selective cation channel that is a propagator of painful signaling that is activated by a variety of stimuli as well^[55]. TRPA1 is mainly expressed in small diameter peptidergic nociceptors of the DRG, nodose and trigeminal ganglia along with TRPV1. TRPA1 has also been shown to be extensively expressed in enterochromaffin cells of colonic myenteric neurons as well as within non-neuronal tissue such as the small intestine and pancreas^[55-57]. Therefore, together with its profile, co-expression pattern and connectivity to afferents that potentially impact the stomach, we hypothesized that TRPA1 would be a potential mediator of ulcer pain.

We found that the TRPA1 antagonist, HC-030031, attenuated abdominal hypersensitivity in the ulcer model. This result is in agreement with a study by Kondo *et al.*^[57] who demonstrated the efficacy of this compound in the context of a noxious gastric distention model. However, these findings are in direct contrast to a recent study by Kojima *et al.*^[58] who examined the effect of the TRPA1 agonist, ASP7663, in a similar model. Surprisingly, they maintained that TRPA1 agonism, not antagonism, represents a rational approach to treating visceral pain and that the analgesic effect of ASP7663 on colorectal distension-induced abdominal pain is mediated by direct desensitization of the TRPA1 channel.

Another potential transducer of noxious signals in the GI tract are ASICs (ASIC 1-3), a family of proton-gated sodium channels expressed within primary afferent neurons. Since a positive correlation between pain and local acidity has been reported and protons trigger inward currents through sodium channels in visceral sensory neurons causing hyper-excitability^[21,59,60], we were surprised that amiloride did not attenuate the referred ulcer pain at any of the doses tested. Another study by Jones *et al.*^[61] examining the effect of amiloride on afferent fiber sensitivity to circumferential stretch of the colon also failed to see efficacy. The authors suggested that different ASIC subunits may exert opposing effects on mechanosensation. Likewise, different subunits may be involved in mechanical hypersensitivity of the colon as shown in a study using ASIC3 knockout mice^[62] and this too may be reflective of the enhanced tactile sensitivity observed following dosing of the non-selective blocker amiloride to naïve animals and its lack of efficacy in the ulcer model.

Voltage-gated sodium channels have also received a significant amount of attention in recent years for their diverse role in pain. Given this, we investigated the role of the non-selective sodium channel blocker, carbamazepine, in the ulcer model. Unlike a prior efficacy report^[63], we found carbamazepine produced a positive signal when administered at a high dose, 100 mg/kg. However, it is difficult to conclude if this was an efficacious response or a mixed result driven by the sedative properties of this molecule. As expected, carbamazepine also affected other measures of neurological function, such as rotarod performance and innate tactile threshold, thereby complicating interpretation of the reflexive endpoint used. Nonetheless, sodium channels appear to be important in pain as they have been causally linked to human conditions like IBS^[64]. Notably, a number of selective sodium channel blockers targeting Nav 1.7 and Nav 1.8 in the periphery have entered clinical development^[65]. Given the expression pattern of these targets as well as their selectivity profiles, one would expect that the not so distant future will provide novel sodium channel blockers that may alleviate conditions associated with visceral hypersensitivity although the discovery and

developmental path for these compounds thus far has been very challenging.

Lastly, we examined whether linaclotide (Linzess™), a first-in-class, orally administered synthetic peptide of the guanylin peptide family, would produce an efficacious response in the ulcer model^[33]. Linaclotide, a GC-C agonist, and its active metabolite are purported to bind to GC-C to act locally within the GI lumen to elevate cGMP. Mechanistically, Silos-Santiago *et al.*^[66] suggest that the cGMP significantly decreases the firing rates of sensitized afferent neurons in response to mechanical stimuli to lessen the pain response. In agreement with this study, we found that linaclotide was efficacious in attenuating ulcer pain when dosed 3-10 µg/kg. Surprisingly, however, the maximum effect was observed when linaclotide was administered at the lowest dose, 3 µg/kg. Although we would not have predicted this result, this finding did align with findings published previously by Eutamene *et al.*^[67] using the colorectal distension model. Similarly, this study showed that higher doses of linaclotide, 30 µg/kg, did not affect colorectal hypersensitivity, while lower doses did attenuate it. Interestingly, IBS clinical trial results examining the efficacy of linaclotide in patients confirmed these preclinical findings as well such that the FDA approved package insert for this compound states that continued abdominal pain (7% vs 5% placebo) is a possible side effect of therapy (www.linzesshcp.com). Although the mechanism for this paradoxical effect is unclear, it could be related to the physiochemical-pharmacokinetic properties of the molecule. Linaclotide has no oral bioavailability when given at efficacious doses; it is purported to act locally within the GI tract. However, with increasing dosage, the PK of linaclotide is documented to be non-linear. So, it is conceivable that such reduced anti-hyperalgesic effects at higher doses may be attributable to a corresponding loss of pharmacological specificity^[67].

Visceral hypersensitivity underlies the pain experienced by patients presenting with various GI-related disorders. Although there is an unmet need to model visceral pain pre-clinically, this can be very challenging given the unique sensory innervation of visceral organs as well as our incomplete understanding of disease etiology^[68]. Nonetheless, given the characteristics of visceral pain, both favorable (*i.e.*, not produced by noxious stimuli) and unfavorable (*i.e.*, pain is referred, diffuse, poorly localized)^[27], we have demonstrated that the ulcer model is uniquely sensitive for assessing the effects of compounds directed against several well-known pain mechanisms. Furthermore, while we think this model is well-suited for drug discovery namely because of its ease and its reliability in performing stringent pharmacological evaluation (*i.e.*, dose-response data), it does have its limitations. For one, it is an acute model of visceral pain. While acute visceral pain is burdensome, the unmet need lies with the undulating hypersensitivity that persists from chronicity like in IBS. Also, most

discovery groups perform their pain studies in rats, so consistency with regard to species selection does differ as this model was validated in mice. Lastly, functional GI disorders represent a heterogeneous group of disorders that include genetic and environmental contributors. Notably, these factors are difficult to recapitulate in animals. While these limitations are well-recognized, together with its clinical relevance, we believe that the NSAID-induced gastropathy model may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts for this unmet need.

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COMMENTS

Background

Non-steroidal anti-inflammatory drugs (NSAIDs) and salicylates are ulcerogenic and therefore, chronic use can exacerbate existing gastric injury or lead to new ulcer formation. For these and other patients, it has been hypothesized that persistent or unresolved visceral pain, despite the etiology, may be due to aberrations in primary afferent function or hypersensitivity, peripheral sensitization, and/or psychological/genetic abnormalities.

Research frontiers

With this in mind, the authors characterized the pain associated with gastric ulceration. By combining a clinically relevant stomach ulcer model with a predictive behavioral endpoint, the authors investigated some potential mechanisms producing visceral hypersensitivity.

Innovations and breakthroughs

Pain is a characteristic feature of many chronic disorders affecting the gastrointestinal (GI) tract. In this study the authors measured the pain associated with NSAID-induced gastropathy and then investigated relevant pharmacological mechanisms underlying its development and maintenance.

Applications

The authors believe that the NSAID-induced gastropathy model may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts for this unmet need.

Peer-review

In this paper, the authors used the indomethacin to create NSAID-induced Gastropathy mouse model, and then investigated the pharmacological role of opioid and guanylate cyclase C receptors as well as TRPs, ASICs and sodium channels on this translatable model of visceral hypersensitivity. In summary, this study has demonstrated that the NSAID-gastropathy model has the potential to efficiently triage molecules with pain-attenuating properties for their utility in GI disorders that include pain as a hallmark symptom, and may help uncover additional targets contributing to persistent GI pain of ill-defined etiology and further advance future drug discovery efforts. The overall structure of the manuscript is complete, and all figures are necessary and appropriate. The study has a certain significance and it may possibly help researchers to develop further studies.

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Basic Study

High yield reproducible rat model recapitulating human Barrett's carcinogenesis

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Abstract

AIM

To efficiently replicate the biology and pathogenesis of human esophageal adenocarcinoma (EAC) using the modified Levrat model of end-to-side esophagojejunostomy.

METHODS

End-to-side esophagojejunostomy was performed on rats to induce gastroduodenoesophageal reflux to develop EAC. Animals were randomly selected and serially euthanized at 10 ($n = 6$), 17 ($n = 8$), 24 ($n = 9$), 31 ($n = 6$), 38 ($n = 6$), and 40 ($n = 6$) wk postoperatively. The esophagi were harvested for downstream histopathology and gene expression. Histological evaluation was

completed to determine respective rates of carcinogenic development. Quantitative reverse transcription-polymerase chain reaction was performed to determine gene expression levels of *MUC2*, *CK19*, and *CK20*, and results were compared to determine significant differences throughout disease progression stages.

RESULTS

The overall study mortality was 15%. Causes of mortality included anastomotic leak, gastrointestinal hemorrhage, stomach ulcer perforation, respiratory infection secondary to aspiration, and obstruction due to tumor or late anastomotic stricture. 10 wk following surgery, 100% of animals presented with esophagitis. Barrett's esophagus (BE) was first observed at 10 wk, and was present in 100% of animals by 17 wk. Dysplasia was confirmed in 87.5% of animals at 17 wk, and increased to 100% by 31 wk. EAC was first observed in 44.4% of animals at 24 wk and increased to 100% by 40 wk. In addition, two animals at 38-40 wk post-surgery had confirmed macro-metastases in the lung/liver and small intestine, respectively. *MUC2* gene expression was progressively down-regulated from BE to dysplasia to EAC. Both *CK19* and *CK20* gene expression significantly increased in a stepwise manner from esophagitis to EAC.

CONCLUSION

Esophagojejunostomy was successfully replicated in rats with low mortality and a high tumor burden, which may facilitate broader adoption to study EAC development, progression, and therapeutics.

Key words: Esophageal adenocarcinoma; Gastroesophageal reflux disease; Levrat; Esophagojejunostomy; Experimental rat model; Mucin genes; Cytokeratins

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Core tip: The current study reports refined surgical techniques with improved tumor burdens for the modified Levrat model of end-to-side esophagojejunostomy in a rat for future *in vivo* studies of esophageal adenocarcinoma (EAC). For the first time, the model was established with significantly reduced mortality and morbidity and further validated through evaluation of conserved EAC disease progression markers, such as mucin and cytokeratins. The reported approach will allow for broader adoption of the model to allow for greater understanding of the complete disease progression spectrum from Barrett's esophagus to metastatic EAC and aid in the development of novel therapeutics.

Matsui D, Omstead AN, Kosovec JE, Komatsu Y, Lloyd EJ, Raphael H, Kelly RJ, Zaidi AH, Jobe BA. High yield reproducible rat model recapitulating human Barrett's carcinogenesis. *World J Gastroenterol* 2017; 23(33): 6077-6087 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6077.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6077>

INTRODUCTION

Esophageal adenocarcinoma (EAC) incidence has increased dramatically in the western world in recent decades, with a 600% increase seen in the United States since the mid-1970s^[1]. Despite recent advances in the development of multimodality patient-centered care plans, EAC still carries a poor prognosis with an overall 5-year survival rate of less than 15%^[2]. Due to the increasing incidence and lethality of EAC, a better understanding of the underlying cancer biology and improved methods for prevention and treatment are urgently needed.

Barrett's esophagus (BE) is induced by chronic gastroesophageal reflux disease (GERD) and is a premalignant precursor to the progression of dysplasia and EAC^[3,4]. GERD stimulates the replacement of squamous epithelium of the distal esophagus by intestinal-type metaplastic columnar epithelium, pathologically characterized by goblet cells and the expression of intestinal and differentiation markers, such as mucin genes (*MUC2*, *MUC5AC*), cytokeratins (CK7, CK20) and villin^[5-9]. Furthermore, several studies have shown that transcription factors involved in the establishment of tissue differentiation, such as CDX2 and SOX2, play a key role in the development of BE and precede morphological changes^[10,11]. Although there is great interest the underlying biology of BE carcinogenesis, the exact molecular mechanisms involved in the longitudinal progression to EAC have not been fully elucidated. Therefore, molecular biomarkers that are dysregulated across the progression spectrum would have significant clinical utility for early detection, risk identification, prognosis and therapeutic intervention.

Well-established small animal models efficiently recapitulate human *de novo* disease progression at the histological and molecular levels, providing clinical utility for development of effective treatment strategies. In 1962, Levrat *et al.*^[12] reported a surgical model of esophagoduodenostomy (ED) in rats to induce chronic gastroduodenal reflux. This model was further refined to demonstrate that ED with or without gastrectomy, esophagojejunostomy (EJ) with or without gastrectomy, and pancreatoco-esophageal anastomosis could recreate disease progression to EAC without the administration of exogenous carcinogens^[13-18]. Additionally, the study of gene expression profiles from BE to EAC confirmed significant homology between human and rat disease^[19]. The modified Levrat model of end-to-side EJ with vagal nerve preservation to induce EAC through chronic gastroduodenoesophageal reflux (GDER) currently represents the gold standard of the model and has been extensively utilized to evaluate EAC disease progression and novel therapeutics. Therefore, this surgical approach provides a representative and translatable model for the study of reflux-induced carcinogenesis and downstream genetic alterations associated with EAC.

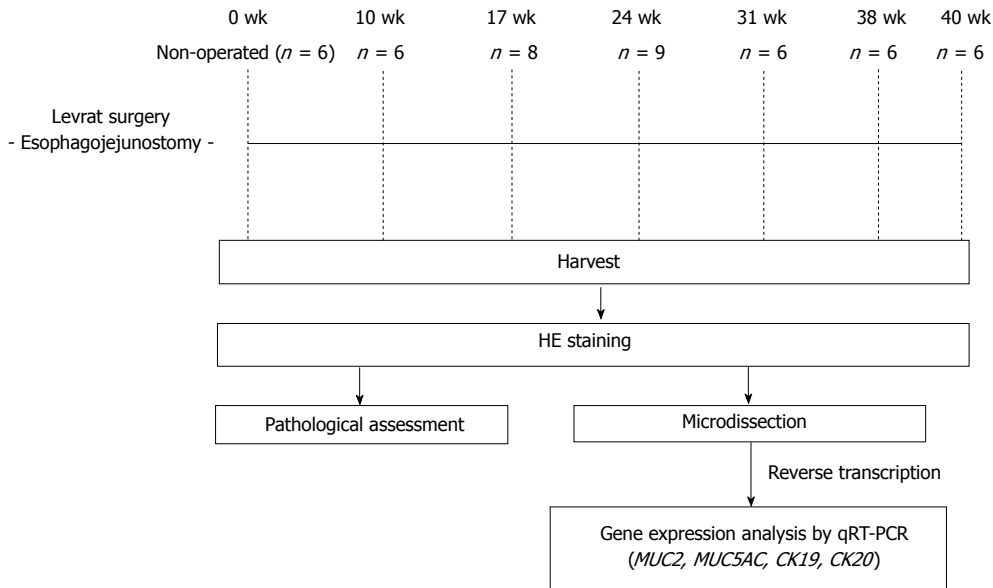


Figure 1 Study schema representing the major steps in the experimental design. qRT-PCR: Quantitative reverse transcription-polymerase chain reaction.

Through our extensive use of the modified Levrat model over recent years to study EAC, we have successfully optimized our protocols to maximize tumor burden and minimize mortality. The aim of the present study was to describe efficient use of the modified Levrat model through improved surgical techniques, perioperative care, management of complications, and analysis of molecular profiles. We feel the successful broader implementation of a reliable and replicable model that mimics human disease will enable other laboratories to reproduce it effortlessly and advance further research into the pathophysiology and biology of EAC.

MATERIALS AND METHODS

Ethics statement

This study was conducted with approval from the Institutional Animal Care and Use Committee of Allegheny General Hospital in Pittsburgh, Pennsylvania under Protocol #992. All animals received humane care in compliance with the standards set forth in "The Guide for the Care and Use of Laboratory Animals." All animals were weighed weekly and euthanized if acute decompensation occurred prior to study endpoint.

Experimental design

The cohort for this study was randomly preassigned from a larger protocol of 225 animals. Mortality and morbidity were calculated from the larger sample set to proportionally reflect the range and incidence rates of causes across sub-studies. The modified Levrat surgery of end-to-side EJ was performed on 200 to 250 g 6-wk to 8-wk-old male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) to induce chronic GDER and the subsequent spectrum of *de novo* premalignant

lesions leading to EAC formation. Animals were randomly selected for serial euthanasia at 10 ($n = 6$), 17 ($n = 8$), 24 ($n = 9$), 31 ($n = 6$), 38 ($n = 6$), and 40 ($n = 6$) wk postoperatively. Additionally, six animals served as controls and were harvested with no surgical intervention. The higher effective n at 17 and 24 wk is reflective of animals that were prematurely sacrificed from the original designated time point due to health considerations. Pathological assessment was performed on all harvested esophageal specimens stained with hematoxylin and eosin (HE). Tissue samples were further evaluated for *MUC2*, *MUC5AC*, *CK19*, and *CK20* gene expression using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to compare each level of disease progression. These markers were selected based on known roles in human esophageal carcinogenesis. Study schema of experimental design is represented in Figure 1.

Modified levrat surgical model

Preoperative management: Prior to intervention, animals were acclimatized for at least one week and housed on a 12-h alternating light-dark cycle. Standard feed included a solid pellet diet and free access to tap water. One day prior to surgery, rats were provided with a gel-based diet (BioServ, Flemington, NJ; # S5769) to encourage stomach clearing to reduce the risk of anastomotic leak. Animals were nil per os (NPO) for 2–4 h before surgery. For sedation, animals were placed in an acrylic anesthetizing chamber for induction with 5% isoflurane and transferred to a nose cone mask for maintenance with 2% isoflurane in 1 mL/L of oxygen. The anesthetized rats were then placed on a circulating water heating bed to maintain adequate body temperature during surgery and were given prophylactic ketoprofen (3 mg/kg) and

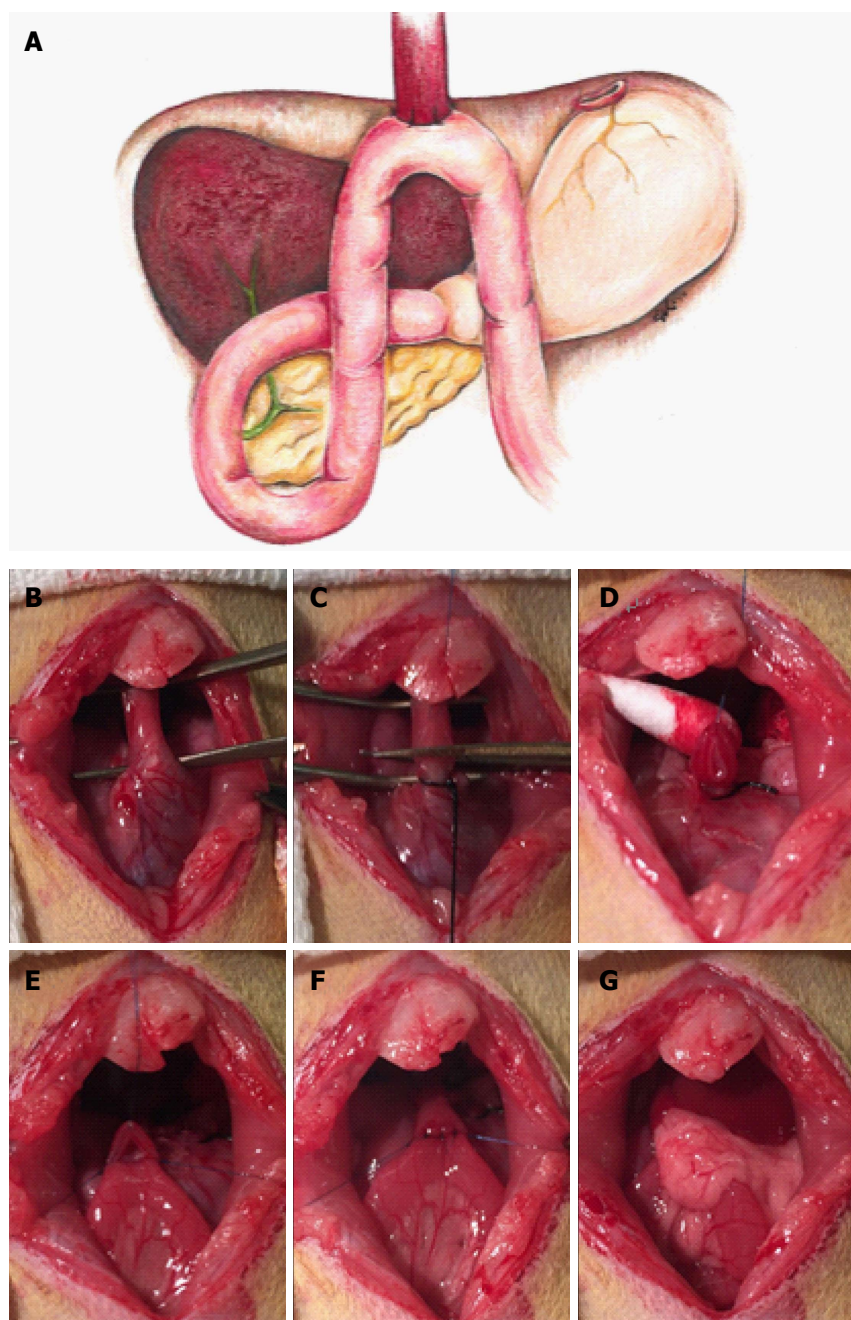


Figure 2 Diagram of modified Levrat model and images of the main steps of the surgical procedure. A: Illustration of end-to-side esophagojejunostomy with gastric preservation; B: Esophagus is mobilized, preserving the left and right vagus nerves; C: Esophagus divided from the stomach after ligation of gastroesophageal junction; D: Anterior esophageal suture placed through muscle and mucosa layers, allowing proper visualization of mucosa; E: Anterior and lateral esophageal sutures intact, creating visualization of the lumen; F: Completed anastomosis between the distal esophagus and jejunum with eight interrupted full-thickness sutures; G: Anastomosis returned to anatomical location wrapped in omentum.

enrofloxacin (5 mg/kg) immediately prior to surgery.

Surgical techniques: All animals underwent an end-to-side EJ with gastric preservation through upper midline abdominal incision. The esophagus was mobilized, preserving the left and right vagus nerves (Figure 2B), and the gastroesophageal junction was ligated using 3-0 silk. A temporary 7-0 prolene suture (Ethicon, Somerville, NJ, United States) was delicately threaded through muscle layer only of the

distal esophagus 1 cm above the silk knot to prevent retraction of the esophagus through the diaphragm. Care was taken to minimize tension on the esophagus to prevent recoiling of the mucosal layer into the lumen, and the esophagus was cut just above the gastroesophageal junction (Figure 2C). A loop of jejunum was identified 4 cm distal to the ligaments of Treitz and 3 mm jejunostomy was made using a #11 surgical blade. Residual intestinal fluid in the immediate area was drained using a sterile swab to

minimize risk of postoperative abscess formation and infection. An end-to-side anastomosis was constructed between the distal esophagus and jejunum in an antecolic manner with eight interrupted full-thickness 7-0 prolene sutures. Special care was taken to include the esophageal mucosa to ensure adequate mucosal-to-mucosal apposition (Figure 2D-F). The first 5 sutures were placed on the anterior wall, and the entire anastomosis was carefully flipped 180° to expose the posterior wall. Before placing the posterior sutures, patency of the proximal and distal lumen was confirmed. The final 3 sutures were placed, and the anastomosis was returned to anatomical position. This delicate flip allowed all suture knots to remain external to the lumen and minimized anastomotic failure due to postoperative obstruction. The omentum was wrapped around the completed anastomosis to prevent anastomotic leakage (Figure 2G), and the celiotomy was closed using 4-0 vicryl (Ethicon, Somerville, NJ, United States). All rats received a 25-30 mg/kg bolus of Lactated Ringer's Solution (LRS), were placed on 100% oxygen until awake, and placed in a cage with a raised wire mesh grate to eliminate direct exposure to obstructive bedding. Rats remained NPO for at least 1 h postoperatively to prevent aspiration.

Postoperative management: For the duration of the study, postoperative animals were housed in cages with raised grates to eliminate access to bedding. Any ingestion of foreign material post-surgery results in anastomotic failure and leak or obstruction. Durable plastic huts were provided as enrichment, and rubber stoppers on water bottles were continuously monitored for integrity. Animals continued to receive daily injections of LRS, ketoprofen (3 mg/kg), and enrofloxacin (5 mg/kg) until postoperative day 3. Additionally, all rats were placed on a 10-d diet modification plan designed to gradually shift from liquid diet to full solid diet, in an effort to facilitate proper healing of the anastomosis, minimize aspiration-related complication, and encourage feeding after surgery. On postoperative days 0 to 3, rats received a nutritional diluted liquid supplement diet (Ensure; Abbot, Columbus, OH, United States) with acetaminophen (Tylenol®) for pain control. Animals were then transitioned to a gel diet (BioServ, Flemington, NJ; # S5769) (days 4-6), followed by a mushed pellet diet (days 7-9), and then regular solid pellet diet on postoperative day 10. The days on a specific modified diet were extended by 1-2 d if the animal seemed slow to recover from surgery. During the postoperative window, all rats were individually housed to closely monitor health, consumption, and stool production and to reduce chances of wound damage. On day 14, all rats were ear-tagged and pair-housed. At 12 wk, all rats were treated with iron dextran (50 mg/kg, i.m.) every two weeks prophylactically for anemia. All animals received a weight check at least once a week, and

during periods of weight loss, rats were weighed more frequently, and modified diets were provided in a supplemental fashion. Animals having more than 45% weight loss or acute decompensation in the postoperative period were sacrificed, and all remaining rats were euthanized at their respective time points for histological evaluation.

Gross and histological evaluation

Tissue preparation and pathological assessment:

Upon necropsy, the entire esophagus and jejunum, to a length approximately 1 cm distal to the anastomosis was harvested. After the specimen was cut open longitudinally, samples were rinsed in ice-cold phosphate buffered saline to remove debris, oriented to maximize exposure of suspicious areas, and flash frozen in Tissue-Tek Optimal Cutting Temperature (OCT) compound (Sakura Finetek, Torrance, CA; #4583). Next, frozen esophagi in OCT blocks were cut into 5 micron sections using a cryostat (Fisher Scientific, Waltham MA; Microm HM 550) and stained with HE for pathological assessment and gene expression analysis. Two experienced pathology experts independently performed the histological analysis to identify areas of disease. Histological changes were defined on the basis of the following established classification criteria: (1) esophagitis: intraepithelial inflammation, thick basal cell layer, elongated lamina propria papillae, and spongiosis; (2) proliferative hyperplasia: Increased thickness of the squamous epithelium (sometimes hyperkeratotic) with no cellular atypia; (3) BE: Replacement of normal esophageal squamous epithelium with columnar-lined epithelium containing goblet cells; (4) dysplasia: dysplastic squamous cell epithelium with enlarged, atypical nuclei and an increased number of mitotic figures, which may invade lamina propria of the epithelium but does not invade the submucosal layer; and (5) EAC: Mucinous, dysplastic glandular cell growth with both atypia and invasion through the basement membrane.

Gene expression analysis by PCR

In order to evaluate the expression of a subset of four biomarkers at each esophageal disease level, qRT-PCR was performed on macrodissected esophageal tissues, including 3 normal esophageal epithelium (controls), 5 esophagitis, 5 BE, 11 dysplasia, 19 EAC and 2 EAC (primary profiled) with metastasis. Briefly, two experienced pathology experts independently confirmed areas of the highest disease for each sample and marked HE slides for macrodissection. Based on the marked areas, 200 µmol/L of tissue was macrodissected using a cryostat, and special care was taken to ensure all collections were highly representative of the disease states. RNA was isolated from the tissue using a miRNeasy kit (Qiagen, Valencia, CA; #217004), and a reverse transcription reaction was performed using a RT² first strand kit (Qiagen,

Table 1 Breakdown of histological findings of each time point after esophagojejunostomy *n* (%)

Histology	Postoperative wk						
	0 wk	10 wk	17 wk	24 wk	31 wk	38 wk	40 wk
	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 8	<i>n</i> = 9	<i>n</i> = 6	<i>n</i> = 6	<i>n</i> = 6
Esophagitis	0 (0)	6 (100)	8 (100)	9 (100)	5 (83.3)	5 (83.3)	6 (100)
Proliferative hyperplasia	0 (0)	6 (100)	8 (100)	9 (100)	6 (100)	6 (100)	6 (100)
Barrett's esophagus	0 (0)	1 (16.7)	8 (100)	8 (88.9)	6 (100)	6 (100)	5 (83.3)
Dysplasia	0 (0)	0 (0)	7 (87.5)	8 (88.9)	6 (100)	6 (100)	6 (100)
Adenocarcinoma	0 (0)	0 (0)	0 (0)	4 (44.4)	4 (66.7)	5 (83.3)	6 (100)
Metastasis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16.7)	1 (16.7)

Valencia, CA, United States; #330401), according to the manufacturer's protocol. qRT-PCR was performed with the RT² SYBR Green ROX qPCR MasterMix (Qiagen, Valencia, CA, United States; #330523) in a total volume of 25 μ L using the following RT² Primer Assays: MUC2 (Qiagen, Valencia, CA, United States; #PPR69984A), MUC5AC (Qiagen, Valencia, CA, United States; #PPR59660B), CK19 (Qiagen, Valencia, CA, United States; #PPR44322A) and CK20 (Qiagen, Valencia, CA, United States; #PPR44539A). Real-time PCR reactions were conducted at 95 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s, using a StepOnePlus real-time quantitative system (Applied Biosystems; Carlsbad, CA, United States). Raw data was exported from the real-time instrument software and relative gene expression was calculated using the $\Delta\Delta$ -Ct method. B-Actin (Qiagen, Valencia, CA, United States; #PPR0650C-200) and RPLP1 (Qiagen, Valencia, CA, United States; #PPR42363C-200) were selected as endogenous controls. All samples were normalized against pathologically confirmed normal squamous esophagus and run in technical triplicates.

Statistical analysis

Statistical analysis was conducted by a biomedical statistician using SPSS software (IBM, Armonk, NY, United States; Version 23). The gene expression levels in normal squamous esophagus, esophagitis, BE, dysplasia, and EAC tissues were compared by an independent 2-tailed *t* test to identify significant differences in expressions. A *P* < 0.05 was considered to be statistically significant.

RESULTS

Surgical model and outcomes

Fifteen percent of the operated animals died before the intended endpoint of the experiment, and necropsies were performed on all euthanized and found dead animals to identify the cause of death. Of these, 23.5% animals died within 2 wk post-surgery due to surgical and procedure-related complications, such as anastomotic leaks and continuous gastrointestinal hemorrhage. An additional 26.5% of the animal deaths were between 2-10 wk following surgery, with the major cause of death being stomach ulcer

perforation. The remaining 50% of animal deaths were 10 wk after surgery, and the major causes of mortality were attributed to respiratory infection secondary to aspiration, stomach ulcer perforation, and obstruction due to tumor or late anastomotic stricture. Overall, the anastomotic leak rate for the study was 2.2%. A randomized cohort of 47 animals was preselected and utilized for histopathology and gene expression. The effective numbers of rats examined for study endpoints were as follows: non-operated (*n* = 6), 10 wk (*n* = 6), 17 wk (*n* = 8), 24 wk (*n* = 9), 31 wk (*n* = 6), 38 wk (*n* = 6), and 40 wk (*n* = 6) after surgery.

Histological findings

The results of histological findings for each disease level at each time point are shown in Table 1. All animals that underwent EJ showed histological features of esophagitis and proliferative hyperplasia from 10 wk following surgery (Figure 3B). BE was observed as early as 10 wk (16.7%; 1/6 animals) following surgery, and 100% of animals at 17, 31, and 38 wk showed the presence of BE (Figure 3C). Dysplasia was first recognized at 17 wk post-surgery (87.5%; 7/8 animals), and the incidence increased over time to 100% at 31-40 wk (Figure 3D). EAC was first observed at 24 wk (44.4%; 4/9 animals) post-surgery and sequentially increased to 100% at 40 wk. All of the neoplastic cases were localized to the esophagus just above the esophagojejunal anastomosis. Five of 6 (83.3%) EAC cases at 40 wk were well-differentiated mucinous carcinomas (Figure 3E). In addition, two animals at 38-40 wk post-surgery had confirmed macro-metastases in the lung/liver and small intestine, respectively (Figure 4).

Gene expression analysis

The relative *MUC2* gene expression was highest in BE, and it was progressively down-regulation across the BE-dysplasia-adenocarcinoma spectrum. When compared to normal squamous esophagus, BE, dysplasia, and EAC had significantly higher expression levels of *MUC2* (*P* = 0.017, 0.005 and < 0.001, respectively). Additionally, there was significant difference in *MUC2* gene expression between esophagitis and BE (*P* = 0.028) (Figure 5A). The relative *MUC5AC* gene expression was highest in EAC and lowest in BE among esophageal disease types. Esophagitis, dysplasia,

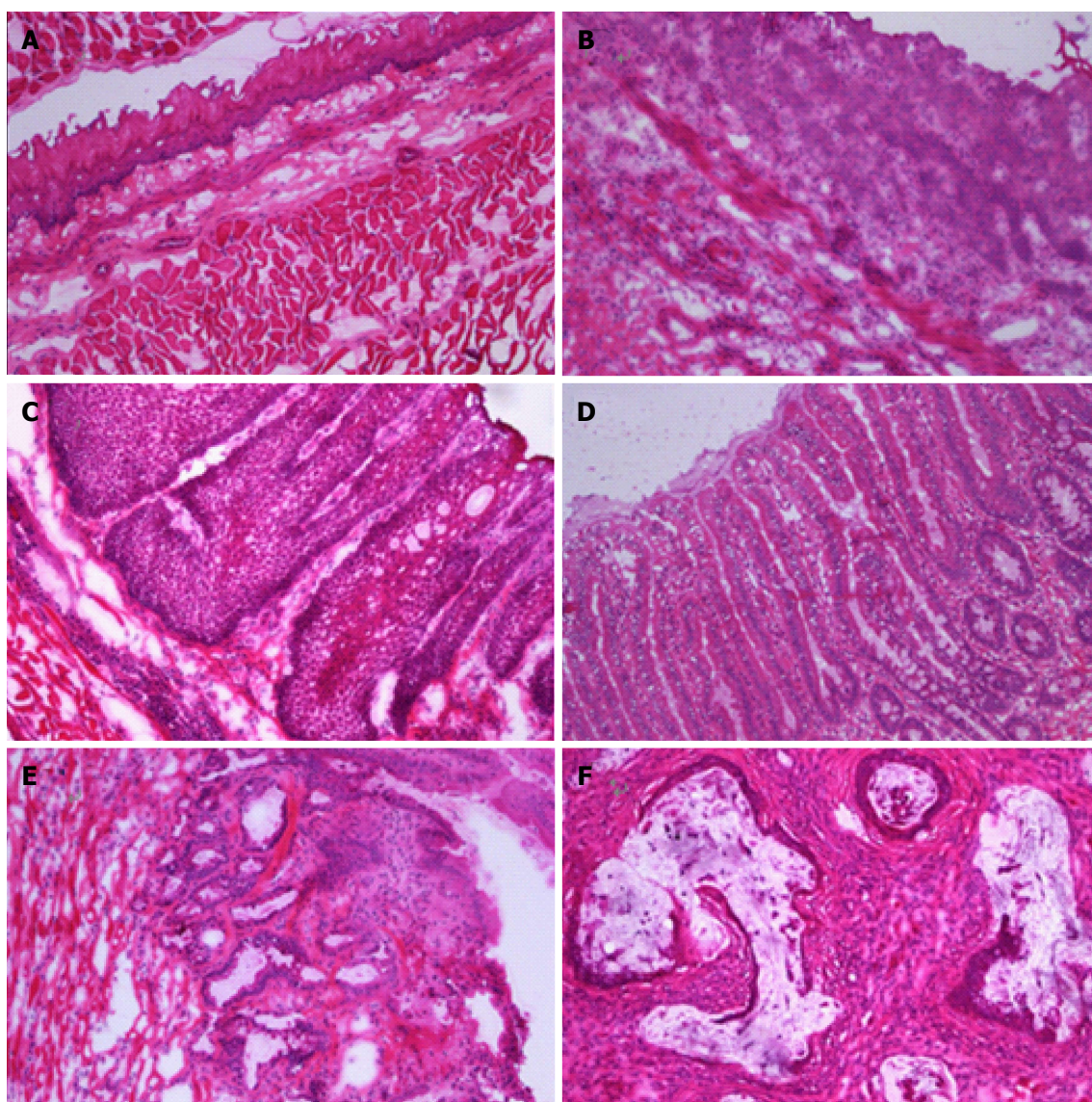


Figure 3 HE staining of esophageal disease progression ($\times 10$). A: Normal rat esophagus; B: Esophagitis; C: BE with goblet cells; D: Dysplasia; E: EAC. EAC: Esophageal adenocarcinoma; BE: Barrett's esophagus.

and EAC had significantly higher expression levels of MUC5AC, compared to normal squamous esophagus ($P = 0.03, 0.021, \text{ and } 0.029$, respectively) (Figure 5B). Both *CK19* and *CK20* gene expression increased in a stepwise manner from esophagitis to EAC. All four histological stages (esophagitis, BE, dysplasia, and EAC) had significantly higher *CK19* and *CK20* expression levels than normal squamous esophagus (*CK19*: $P = 0.019, 0.015, < 0.001$ and < 0.001 , respectively; *CK20*: $P = 0.047, 0.018, 0.001$ and < 0.001 , respectively) (Figure 5C and D).

DISCUSSION

The present study describes a detailed surgical technique and efficient perioperative management for the modified Levrat surgery of end-to-side esophagojejunostomy with gastric preservation. Implementation of the outlined standards provided maximal utilization

of the model to produce a high tumor burden balanced with minimal mortality. Additionally, successful management of perioperative complications allowed us to extend the postoperative window to 40 wk to evaluate well-differentiated EAC and metastasis.

The overall mortality rate in this study was 15%, with 23.5% of the total mortality within the first 2 wk post-surgery, resulting in an overall procedure-related mortality of only 3.5%. This rate was lower than that reported by other investigators for the modified Levrat model^[20,21]. Improved and aseptic surgical techniques and perioperative managements may have also contributed to the reduction of operative mortality. Of note, taking special care to include the esophageal mucosa when constructing the anastomosis was essential to obtain adequate mucosal-to-mucosal apposition. Additionally, covering the anastomotic site with omentum minimized the risk of anastomotic leakage post-surgery. A postoperative progressive

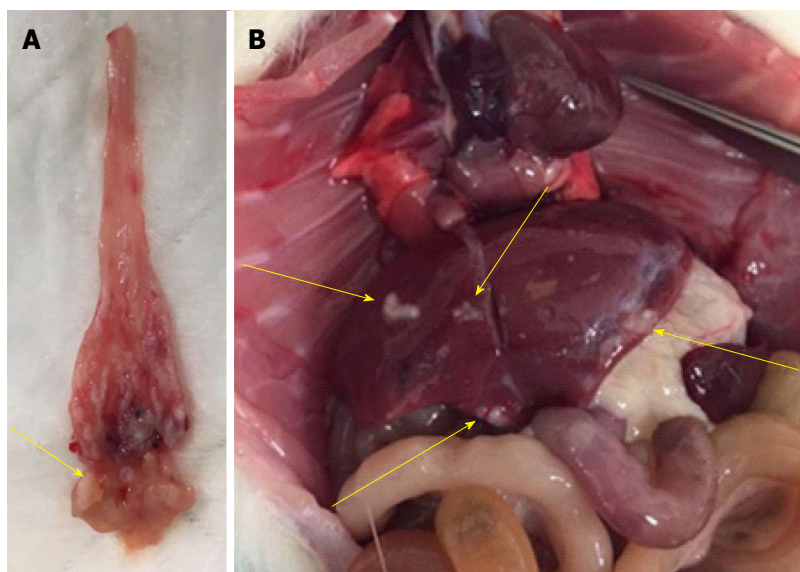


Figure 4 Primary esophageal adenocarcinoma tumor (A) and respective liver metastasis (B), as grossly observed in a 40-wk post-surgical rat.

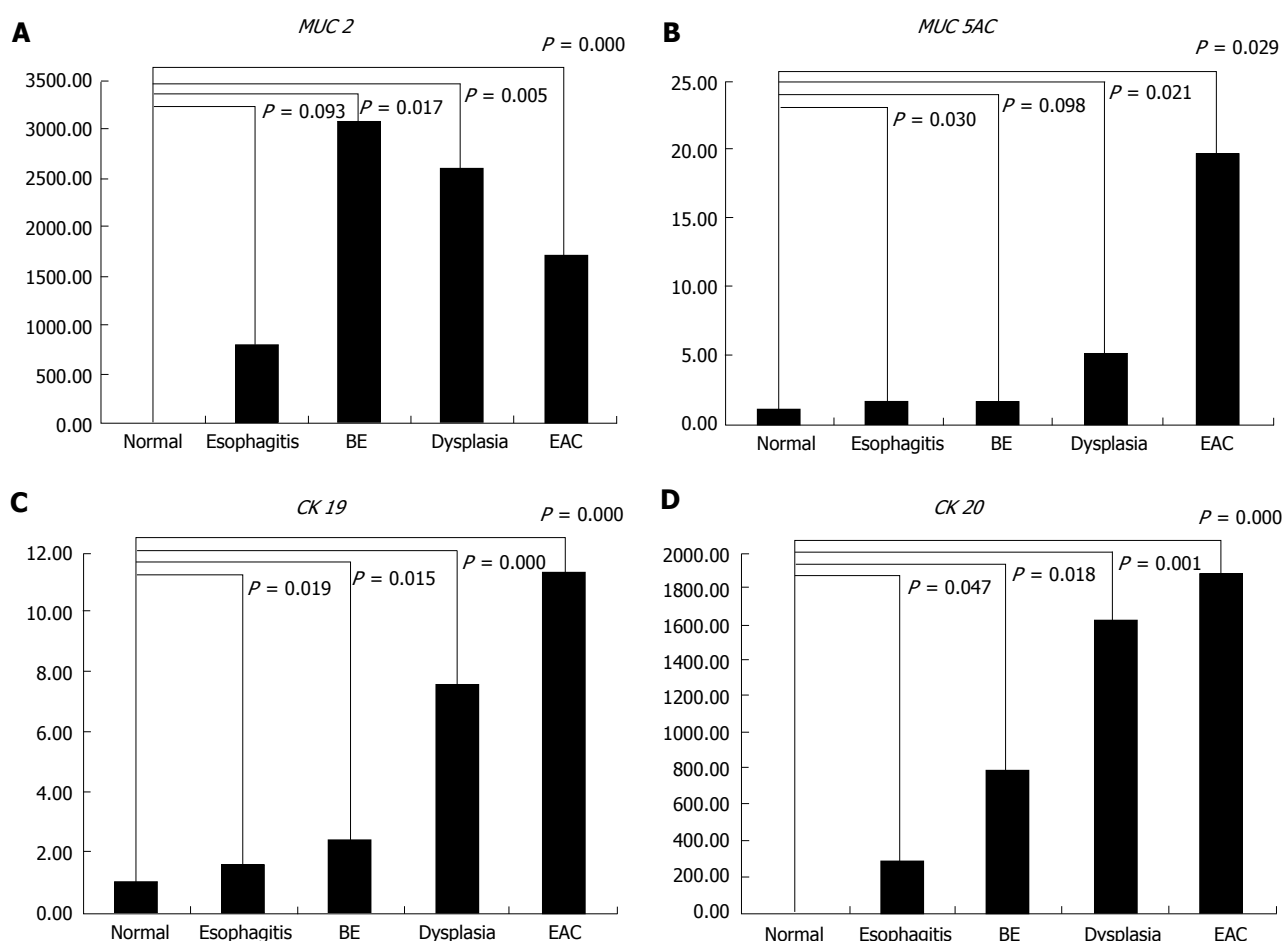


Figure 5 Panel A to D represents the relative gene expression level of *MUC2*, *MUC5AC*, *CK19*, and *CK20* for each esophageal disease level, respectively. BE: Barrett's esophagus; EAC: esophageal adenocarcinoma.

modified diet plan greatly contributed to the protection of the surgical site for the first 10 d after surgery to minimize leak rate. Additionally, supplemental diets provided during periods of weight loss or illness minimized acute morbidity, as anastomotic stricture or tumor growth presented a significant risk factor for

obstruction.

As shown in the present study, the rat reflux model induced by EJ displayed sequential changes in the esophageal epithelium at the site of anastomosis and distal esophagus, similar to those seen in human esophagus. Dysplastic changes were first recognized

near the anastomosis in 87.5% rats at 17 wk post-surgery. Additionally, 83.3% (5/6 animals) and 100% (6/6 animals) of the animals evaluated at 38 wk and 40 wk post-surgery showed histological evidence of EAC at the site of anastomosis with an adjacent area of dysplastic BE, respectively. The overall incidence of EAC reported in this study was higher compared to other previously reported rates of only 17.4% at 30 wk and 74% at 40 wk for the rat surgical reflux model^[22,23]. This difference is likely a direct result of improved surgical techniques and successful management of health complications that allowed for the extension of the perioperative window to 40 wk. Additionally, frequent iron injections may enhance esophageal carcinogenesis by increasing oxidative stress^[24]. In other words, oxidase damage could be a contributing factor in the formation of EAC in the rat reflux model, and may similarly occur in human patients with GERD and iron overload. Moreover, the high incidence of EAC and the presence of distant metastasis at 38-40 wk post-surgery may indicate that the 38-40 wk time point is ideal for the study of EAC progression and metastasis in the modified Levrat model.

Since Levrat and colleagues first described the surgically induced reflux model of esophagitis by performing ED in rats, it has been extensively used to study esophageal carcinogenesis and to evaluate novel preventive and treatment strategies^[25-27]. In our previous studies, we have utilized the modified Levrat's surgical model for studying both chemoprevention and targeted therapy and have demonstrated potent efficacy for multiple cancer mechanistic inhibitors against EAC progression^[20,28,29]. For the first time, we demonstrated macro-metastatic lesions originating from the primary tumors in the model^[30]. Additionally, we further enhanced the utility of the model through combined magnetic resonance imaging (MRI) and small animal endoscopic biopsy to simultaneously track *in vivo* tumor volumes and molecular correlates longitudinally^[31]. With these advancements, the animal model has been highly efficient, not only to develop preventative and treatment strategies, but also to provide a platform to better understand the molecular mechanisms that affect disease progression from inflammation to EAC.

The identification and validation of molecular biomarkers that modulate expression across the progression spectrum may serve as powerful tools for early detection, risk stratification, prognosis, and the development of treatment strategies for EAC. The present study assessed the expression levels of *MUC2*, *MUC5AC*, *CK19*, and *CK20* at each esophageal disease level harvested from surgically induced rat reflux model. Interestingly, compared to normal squamous esophagus and esophagitis, statistically significant *MUC2* expression was highest in BE and then progressively down-regulated through the neoplastic sequence.

Mucin genes are expressed in a site specific manner in the human gastrointestinal tract, and all MUC subtypes are aberrantly expressed in Barrett's metaplasia^[32,33]. Although BE had a low *MUC5AC* expression level in this study, the expression pattern of *MUC2* was consistent with reported human esophageal literature^[6,7,34]. Furthermore, this study demonstrated a relative stepwise increase in expression of *CK19* and *CK20* across the EAC progression. This result may support the possible role of these markers for early detection and risk stratification.

Limitations of the study included small sample size and precision of histological macrodissection. Although the sample set utilized for molecular analysis was small, a quantitative RT-PCR approach was utilized to determine gene expression. Additionally, an inherent limitation of macrodissection included possible inadvertent inclusion of heterogenous tissue that could conceal a specific cell-type signature. Therefore, further investigation with a larger sample size, or applying a microdissection technique to isolate a highly-enriched pure cell population may be beneficial.

In summary, the present study displays improved surgical technique and successful perioperative management of the modified Levrat model to enhance the value in context of a low mortality rate and consistently higher disease burden. This will enable broader adoption of the model to facilitate further research into the pathophysiology and biology of EAC. We further demonstrated a unique expression pattern of *MUC2*, *CK19*, and *CK20* that was relevant to human EAC progression, reinforcing the possible utility as conserved diagnostic molecular markers of BE and EAC.

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COMMENTS

Background

Esophageal adenocarcinoma (EAC) is an extremely lethal disease with an overall survival rate of less than 20%. Small animal models are required to efficiently replicate and study the biology and pathogenesis of human EAC.

Research frontiers

Currently, the treatment options for EAC are outdated, reflected by lack of improvement in patient outcomes over the last three decades, thereby establishing the need for the development of new and improved therapeutic paradigms. The modified Levrat surgery in a rat model has been previously utilized successfully to accelerate development of such agents for chemoprevention, but significant improvements are required to expand the applicability for treatment of established-disease.

Innovations and breakthroughs

These findings report a validated highly replicable protocol to study EAC carcinogenesis and subsequent utilization for treatment efficacy studies. For the first time, the authors demonstrate a significantly reduced mortality rate and increased tumor burden. Additionally, they further validate the translatable nature of the model through gene expression analysis of conserved markers of human Barrett's carcinogenesis, such as mucin and cytokeratin.

Applications

The reported methodology will allow for more efficient utilization of the model to study EAC disease progression and treatment options, which in turn will be translated to clinical settings to improve patient prognosis.

Peer-review

This is a well-designed basic study. Authors in this study generated modified End-to-side esophagojejunostomy (EJ) rat model. Using this model, authors determine respective rates of carcinogenic development and gene expression levels of *MUC2*, *CK19*, and *CK20*. In order to better understand the underlying biology and prevent and treat EAC, the modified EJ model generated in the present study and the data obtained are important for understand disease progression spectrum from Barrett's esophagus to metastatic EAC.

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Basic Study

Changes in expression of inhibitory substances in the intramural neurons of the stomach following streptozotocin-induced diabetes in the pig

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Abstract

AIM

Influence of chronic hyperglycemia on chemical coding of enteric neurons in stomach using pig as a model for human diabetic complications.

METHODS

Ten pigs were divided into two groups: diabetic (D group, $n = 5$) and control (C group, $n = 5$). Pigs constituting the experimental group were given streptozotocin (150 mg/kg). Animals were euthanized

six weeks after the induction of diabetes. The samples of stomach were collected from animals of both groups. The cryostat sections were processed for double immunofluorescence staining using primary antisera directed towards pan-neuronal marker (Hu C/D) proteins and/or neuronal isoform of nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP) and galanin (GAL).

RESULTS

In the control group in the myenteric ganglia (MG) of the corpus we have noted $22.28\% \pm 1.19\%$ of nNOS positive neurons, while in diabetic group we have found $40.74\% \pm 2.22\%$ of nNOS immunoreactive perikarya (increase by 82.85 %). In turn in the pylorus we have observed $15.91\% \pm 0.58\%$ nNOS containing neurons in control animals and $35.38\% \pm 1.54\%$ in the diabetes group (increase by 122.37%). In the MG of the antrum and submucosal ganglion (SG) in the corpus hyperglycemia did not cause statistically significant changes. With regard to VIP-positive cell bodies in the antrum MG in the control animals we have noted $18.38 \pm 1.39\%$ and $40.74\% \pm 1.77\%$ in the experimental group (increase by 121.65%). While in the corpus we have observed $23.20\% \pm 0.23\%$ in the control and $30.93\% \pm 0.86\%$ in the diabetes group (increase by 33.31%). In turn in the pylorus VIP positive cells bodies constituted $23.64\% \pm 1.56\%$ in the control group and $31.20\% \pm 1.10\%$ in the experimental group (increase by 31.97%). In the submucosal ganglion in the corpus we have noted $43.61\% \pm 1.06\%$ in the control animals and $37.00\% \pm 1.77\%$ in the experimental group (decrease by 15.15%). Expression of GAL-positive perikarya showed statistically significant changes only in the MG of the antrum and pylorus. In the antrum GAL positive perikarya constituted $26.53\% \pm 1.52\%$ in the control and $36.67\% \pm 1.02\%$ in the experimental animals (increase by 38.22%). While in the pylorus GAL positive neurons in the control group constituted $16.32\% \pm 0.92\%$ and $17.99\% \pm 0.38\%$ in the experimental animals (increase by 10.23%).

CONCLUSION

Our results support the hypothesis that in the course of diabetes, long term episodes of high glucose serum level may influence the chemical phenotyping of enteric neurons.

Key words: Immunohistochemistry; Inhibitory neurons; Streptozotocin; Hyperglycemia; Pig

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Core tip: Our results revealed the neuronal plasticity of enteric neurons within porcine stomach in response to chronic hyperglycemia. We used the pig as a model for human gastrointestinal disorders occurring in people with diabetes. Our study highlights the important role of the enteric nervous system in response to high glucose serum level. We observed a substantial

increase in the expression of nitric oxide, galanin and vasoactive intestinal peptide inside the enteric neurons. Since all of the investigated molecules have inhibitory properties, they may be involved in the impairment of the motor function of the stomach occurring in people with long-term diabetes.

Bulc M, Palus K, Zielonka L, Gajęcka M, Calka J. Changes in expression of inhibitory substances in the intramural neurons of the stomach following streptozotocin-induced diabetes in the pig. *World J Gastroenterol* 2017; 23(33): 6088-6099 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6088.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6088>

INTRODUCTION

Diabetes is one of the most frequently diagnosed endocrinopathies worldwide^[1,2]. Long-term episodes of hyperglycemia often occur during course of diabetes. As a consequence of poorly-controlled glucose serum level, numerous tissue and organ dysfunctions can occur^[3]. The central, peripheral and autonomic neurons are particularly vulnerable to carboxylic stress, which is a consequence of hyperglycemia^[4]. One of the many consequences of autonomic system damage are numerous disorders in the gastrointestinal tract^[5-7]. They can involve any organ in the digestive system, such as the esophagus, stomach, gallbladder tract, pancreas, small and large intestines. Generally, they are referred to as diabetic gastrointestinal autonomic neuropathy^[5,8]. These disorders are not directly life-threatening, but often lead to a significant impairment in the quality of life^[9]. Up to 75% of patients with long-term diabetes experience gastric motor dysfunction, leading to the following symptoms: constipation, nausea, heartburn, post-prandial fullness, abdominal pain as well as diarrhea^[10,11].

In physiological conditions, the enteric nervous system (ENS) regulates the function of the gastrointestinal tract. ENS (called the "enteric brain") is independent of the central nervous system control^[12]. This unique arrangement of neurons is organized in the muscular and submucosal plexuses and can control secretion processes, absorption and gastrointestinal motility^[13]. The functioning of enteric neurons is based on the secretion of a broad spectrum of neurotransmitters, which are structurally and chemically differentiated^[14]. The most useful division of bioactive substances of enteric neurons include two classes of neurons: inhibitory and excitatory^[12,13]. The nitric oxide (NO) produced by enteric neurons is a major non-adrenergic, non-cholinergic (NANC) inhibitory neurotransmitter which mediates smooth muscle relaxation in the gastrointestinal tract and is, therefore, an important factor in the stomach and gut contractility^[14]. Vasoactive intestinal peptide (VIP),

together with NO comprise the primary inhibitory NANC neurotransmitters^[15]. The biological activity of VIP leads to hyperpolarization of the smooth muscle fibers. Moreover, the relaxation function of VIP involves increased production of NO in smooth muscle cells^[14,15]. Consequently, VIP acts as an inhibition factor of gastric emptying and reduced gastric acid secretion. Simultaneously, VIP is involved in the regulation of blood flow in the submucosal layer^[16]. It is also essential that this substance has an effective neuroprotective function. This has been confirmed by the increasing survival of neurons previously damaged by lipopolysaccharide, as well as in the case of axotomy^[17,18]. Galanin (GAL) can play both inhibitory and excitatory functions, depending on the digestive tract region, as well as the animal species. For example, in the dog ileum galanin is an excitatory factor while in the guinea pig it exhibits an opposite action^[19].

Notably, in the field of metabolic disorders, the pig has gained appreciable interest as its metabolic, biochemical and pathophysiological response to diabetes partly mimics that observed in humans^[20-22]. It has been shown that the blood supply of the porcine pancreas is similar to the human pancreas and the number of insulin-producing cells is within a similar range as observed in humans, making it a valuable model for the study of diabetes^[23]. The most suitable and generally accepted pig model of hyperglycemia is streptozotocine-induced type 1 diabetes, where carbohydrate metabolism and pancreatic insulin secretion remain at a very similar level to humans with long-term diabetes^[20]. Thus, taking advantage of its close similarity to humans, we raised the question of how the porcine gastric enteric neurons adapt to hyperglycemia. Therefore, the aim of this study was to provide data on the adaptive changes in chemical phenotyping, including inhibitory substances [vasoactive intestinal polypeptide, galanin and neuronal isoform of nitric oxide synthase (nNOS)] in the gastric enteric neurons of streptozotocin-induced hyperglycemic pigs.

MATERIALS AND METHODS

Animals

Ten juvenile both sex pigs of the White Large Polish breed, weighing from 17.0 kg to 20 kg, were used in this study. The pigs were randomly divided into two groups: diabetic (D group, $n = 5$) and control (C group, $n = 5$) and were housed in cages suitable for pigs. Prior to the experiment initiation, the animals were given one week of acclimatization to observe their general health, to minimize physiological stress and to ensure the proper conduct of the study. All treatment of animals was conducted in compliance with the instructions of the Local Ethical Committee in Olsztyn (Poland) (decision number 13/2015/DTN)

with special attention paid to minimizing any stress reaction.

Chemical induction of diabetes

After acclimatization, hyperglycemia in the diabetic group was induced by use of streptozotocin (STZ) (Sigma-Aldrich, St Louis, MO, United States, S0130) 150 mg/kg of body weight, dissolved in a freshly-prepared disodium citrate buffer solution (pH = 4.23, 1 g streptozotocin/10 mL solution). For this purpose, pigs were anesthetized and the solution was administered *via* intravenous needle inserted into an ear with continuous infusion for approximately 5 min. To avoid nausea and vomiting after streptozotocin injection, animals were fasted for 18 h before the experiment and the control pigs were injected with equal amounts of vehicle (citrate buffer).

General health condition evaluation

The pigs were continuously observed for 24 h after streptozotocin injection. In order to avoid temporary hypoglycemia, 250 mL of a 50% glucose solution per animal was administered intravenously. The pigs received a normal diet throughout the whole time of the experiment twice a day and tap water *ad libitum*. The blood glucose level was measured to confirm diabetes. The blood glucose concentration was estimated using an Aceent-200 (Cormay) biochemical analyzer, with the colorimetric measurement at a wavelength of 510 nm/670 nm. For this purpose, capillary blood from the ear was collected. The plasma glucose level was measured prior to the experiment initiation in both control and experimental groups. The next measurement was made 48 h after the induction of diabetes. Subsequent measurements of glucose levels were monitored weekly until the end of the experiment.

Tissue collection

Six weeks after streptozotocin injection, animals were deeply anesthetized *via* intravenous administration of pentobarbital (Vetbutal, Biowet, Poland) and perfused transcardially *via* the ascending aorta with 4% paraformaldehyde in a 0.1 mol/L phosphate buffer (PB, pH 7.4). The samples were post-fixed by immersion in the same fixative for 1 h, rinsed several times with phosphatase buffer (PB) and then transferred into 30% sucrose solution and stored at 4 °C until sectioning. The tissue blocks were cut in frontal or sagittal planes using a Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 µm and mounted on gelatinized glass slides.

Immunofluorescence procedure

The sections were processed for a double immunofluorescence staining. Briefly, after air-drying at room temperature for 45 min and rinsing in 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4; 3 ×

Table 1 Serum glucose levels

Date	Control group (mmol/L)	SE	Experimental group (mmol/L)	SE
Before streptozotocin injection	5.01	0.10	5.030	0.10
1 wk after streptozotocin injection	5.08	0.10	17.36	0.38
2 wk after streptozotocin injection	4.91	0.18	20.72	0.24
3 wk after streptozotocin injection	5.19	0.06	21.58	0.27
4 wk after streptozotocin injection	5.31	0.12	20.08	0.09
5 wk after streptozotocin injection	4.84	0.32	22.26	1.21
6 wk after streptozotocin injection	5.20	0.10	21.45	1.11

Serum glucose levels in controls and after induction of diabetes by streptozotocin administration (up 1 to 6 wk).

10 min), the sections were incubated in a blocking buffer containing: 10% of normal goat serum (MP Biomedicals, United States), in 0.1 mol/L PBS, 0.1% donkey serum (Abcam, United Kingdom), 1% Triton X-100 (Sigma-Aldrich, United States), 0.05% Thimerosal (Sigma-Aldrich, United States) and 0.01% NaN₃ for 1 h at room temperature to reduce non-specific background staining. Subsequently, after another wash in PBS (3 × 10 min) the sections were incubated overnight at 4 °C with primary antibodies raised in different species and directed towards general neuronal marker Hu C/D proteins (mouse polyclonal: Invitrogen United States; code A-212711:1000 working diluted 1:1000), anti-nNOS antibodies (rabbit polyclonal: Chemicon, Billerica, MA, United States, cat. No. AB 5380; working dilution 1:4000), VIP (rabbit polyclonal; Biomol, Hamburg, Germany, cat. No. VA1285; working dilution 1:6000) and GAL (rabbit polyclonal: Millipore, Billerica, MA, United States, cat. No. AB 2233; working dilution 1:1000). All antibodies were diluted in PBS containing 0.3% Triton X-100 and 1% BSA. On the following day, the sections were rinsed (PBS, 3 × 15 min) and incubated with secondary antibodies (donkey anti-mouse Alexa Fluor 488, 1:1000 Invitrogen United States; code A21202, and donkey anti-rabbit Alexa Fluor 546 1:1000 Invitrogen, United States; code A10040) diluted in PBS containing 0.25% BSA and 0.1% Triton X-100 for 4 h. The sections were then rinsed three times (PBS, 3 × 5 min) and mounted in fluorescent mounting medium (DAKO, Carpinteria, CA, United States). The prepared specimens were viewed and photographed using an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, coupled with a digital monochromatic camera (Olympus XM 10) connected to a PC and analyzed with Cell Dimension software (Olympus, Tokyo, Japan). Standard controls, *i.e.*, pre-absorption of the neuropeptide antisera with appropriate antigen, omission, and replacement of the primary antisera by non-immune sera, were performed to test the antibodies and specificity of the method. The test was performed as follows: sections of the stomach were incubated with "working" dilution of the primary immunoserum, which had

been previously pre-absorbed for 18 h at 37 °C with 20 µg of appropriate purified protein VIP (064-24, Phoenix Pharmaceutical), GAL (026-06, Phoenix Pharmaceutical) and nNOS (N3033, Sigma, St Louis, MO, United States). Additional negative controls, such as omission and replacement of all primary antisera with non-immune sera, were also performed. This procedure completely eliminated specific staining.

Counting of the nerve structures and statistical evaluation

The number of nNOS, VIP and GAL-like immunoreactive (LI) enteric neurons was expressed as a percentage of the total number of Hu C/D positive perikarya. At least 700 Hu C/D labeled cell bodies of intramural ganglia and each part of the stomach were examined. Only neurons with well-visible nucleus were counted. To prevent the double-counting of Hu C/D immunoreactive neurons, the sections were located at least 100 µm apart. Data pooled from all animals groups were statistically analyzed using Statistica 10 software (StatSoft Inc., Tulsa, OK, United States) and were expressed as a mean ± SE. Significant differences were evaluated using Student's *t*-test for independent samples (^a*P* < 0.05, ^b*P* < 0.01, and ^c*P* < 0.001).

RESULTS

Plasma glucose level and physiological status

The baseline plasma glucose level measured in animals before STZ treatment and the development of hyperglycemia were both within standard reference values for the pig (5.01 mmol/L ± 0.10 mmol/L). During the weeks following STZ implementation, a consistent increase in plasma glucose concentration was observed. A significant 3.4 -fold (17.36 mmol/L ± 0.38 mmol/L) increase in glucose level was observed on the 7th day after STZ injection and 4-5 wk after the injection the highest increase (4.4 fold) level of glucose was observed (22.26 mmol/L ± 1.21 mmol/L). In the last week of experiment, the baseline serum glucose level decreased slightly, reaching 21.24 mmol/L ± 1.11 mmol/L. The mean glucose concentration during the experimental period is presented in Table 1.

Despite significant hyperglycemia, none of the diabetic animals showed any abnormalities throughout the entire period of the experiment. Moreover, none of the animals required exogenous insulin injection.

Immunofluorescence

All biologically active substances studied were presented in the investigated area of the stomach (Table 2). In the control group, the nNOS distribution varied and clearly depended on the analyzed area of the stomach (Figure 1A-S and Figure 2A-F). In the myenteric ganglia (MG) of the antrum, the total number of nNOS neurons was 37.12% ± 2.81% (Figure 1A-C), while in the corpus,

Table 2 The average percentage of neurons immunoreactive to bioactive substances used in this study

Stomach part	Control group			Experimental group		
	Antrum	Corpus	Pylorus	Antrum	Corpus	Pylorus
		nNOS ¹			nNOS ¹	
Myenteric ganglia	37.12 ± 2.81	22.28 ± 1.19	15.91 ± 0.58	41.93 ± 2.34	40.74 ^b ± 2.22	35.38 ^c ± 1.54
Submucosal ganglia	-	18.62 ± 1.66	-	-	19.79 ± 1.51	-
		VIP ¹			VIP ¹	
Myenteric ganglia	18.38 ± 1.39	23.2 ± 0.23	23.64 ± 1.56	40.74 ^d ± 1.77	30.93 ^e ± 0.86	31.20 ^a ± 1.10
Submucosal ganglia	-	43.61 ± 1.06	-	-	37.00 ^a ± 1.77	-
		GAL ¹			GAL ¹	
Myenteric ganglia	26.53 ± 1.52	17.73 ± 1.12	16.32 ± 0.43	36.67 ^b ± 1.02	16.51 ± 0.92	17.99 ^a ± 0.38
Submucosal ganglia	-	41.42 ± 0.88	-	-	40.49 ± 0.63	-

¹Relative frequency of particular neuronal subclasses is presented as percent (mean ± SE) of all neurons counted within the ganglia stained for Hu C/D proteins. nNOS, VIP and GAL immunoreactive perikarya in various parts of the porcine stomach under physiological conditions (Control group) and during experimentally induced hyperglycemia (Experimental group). Statistically significant data (^a*P* < 0.05, ^b*P* < 0.01, and ^c*P* < 0.001) in particular animal group are marked by superscripted alphabetical lettering. nNOS: Neuronal isoform of nitric oxide synthase; VIP: Vasoactive intestinal peptide; GAL: Galanin; Hu C/D: Pan-neuronal marker.

the quantity of nNOS positive cells bodies inside the MG was relatively lower 22.28% ± 1.19% (Figure 1G-I). Additionally, in submucosal ganglion (SG) in the corpus, nNOS-LI neurons were estimated at 18.62% ± 1.66% (Figure 2A-C). In the pylorus, in MG about 15.91% ± 0.58% nNOS-LI were observed (Figure 1M-O).

Although experimentally-induced hyperglycemia contributed to the expression of nNOS in enteric neurons, the changes were differentiated. Statistically significant changes were observed in the MG in the corpus from 22.28% ± 1.19% to 40.74% ± 2.22% (increase by 82.85%) (Figure 1J-L) increased of 18.46% and in the pylorus from 15.91% ± 0.58% to 35.38% ± 1.54% (increase by 122.37%) (Figure 1P-S) increased of 19.47%. In contrast, in the MG in the antrum and SG in the corpus, hyperglycemia did not cause statistically significant changes (Figures 1D-F and 2D-F).

The other investigated substance was VIP. As in the case of nNOS, VIP-positive cells were presented in all studied areas, but clear differences were noted between the various regions of the stomach (Figures 2G-L and 3A-S). In the control group the largest number of VIP-positive cells bodies were noted within the SG in the corpus 43.61% ± 1.06% (Figure 2G-I). Definitely fewer VIP-positive perikarya were observed within the MG in the antrum 18.38% ± 1.39% (Figure 3A-C), pylorus 23.64% ± 1.56% (Figure 3M-O) and MG of the corpus 23.20% ± 0.23% (Figure 3G-I).

In the diabetic group, statistically significant changes were observed in all investigated areas. The highest increase in VIP-immunopositive perikarya was noted in the MG inside the corpus from 18.38% ± 1.39% to 40.74% ± 1.77% (increase by 121.65%) (Figure 3J-L). With regard to MG in the antrum and pylorus, the changes in chemical coding were relatively smaller from 23.20% ± 0.23% to 30.93% ± 0.86% (increase by 33.31%) inside to antrum (Figure 3D-F) and from 23.64 ± 1.56% to 30.47% ± 1.10% inside to pylorus, respectively (increase by 31.97%) (Figure

3P-S). However, in the SG of the corpus, a decrease in the number of VIP-LI neurons was noted from 43.61% ± 1.06% to 37.00% ± 1.77% (decrease by 15.15%) (Figure 2J-L).

As in the case of previously studied substances, the distribution of GAL varied and clearly depended on the area of the stomach (Figure 2M-S and 4A-S). In the control group, the largest population of cells were observed in the MG of antrum 26.53% ± 1.52% (Figure 4A-C). In the MG in the area of corpus and pylorus, a comparable number of GAL-positive neurons were observed 17.73% ± 1.12% in the corpus (Figure 4G-I) and 16.32% ± 0.43% in the pylorus (Figure 4M-O). The highest population of GAL-positive perikarya was noted in the SG in the corpus 41.42% ± 0.88% (Figure 2M-O).

In the diabetic group, statistically significant changes were noted only in the MG in the antrum and pylorus, whereas increased expression of GAL-LI in enteric neurons was observed from 26.53% ± 1.52% to 36.67% ± 1.02% in the antrum (increase by 38.22%) (Figure 4D-F) and from 17.73% ± 1.12% to 17.99% ± 0.38% in the pylorus (increase by 10.23%) (Figure 4P-S). However, in the corpus in both the MG and SG, the population of GAL-LI neurons changed in a statistically insignificant manner compared to the control group (Figures 4J-L and 2P-S).

DISCUSSION

Autonomic neuropathy is a commonly occurring complication in poorly-controlled blood glucose level patients. Studies of the development mechanisms of this pathology have been conducted in many research center^[4,5,8,23]. Moreover, different animal models have been used to determinate the exact mechanism of diabetes complications^[24]. Thus, there are doubts as to whether the pig is a suitable model for the study of diabetic autonomic neuropathy occurring in patients with long-term diabetes. Unfortunately,

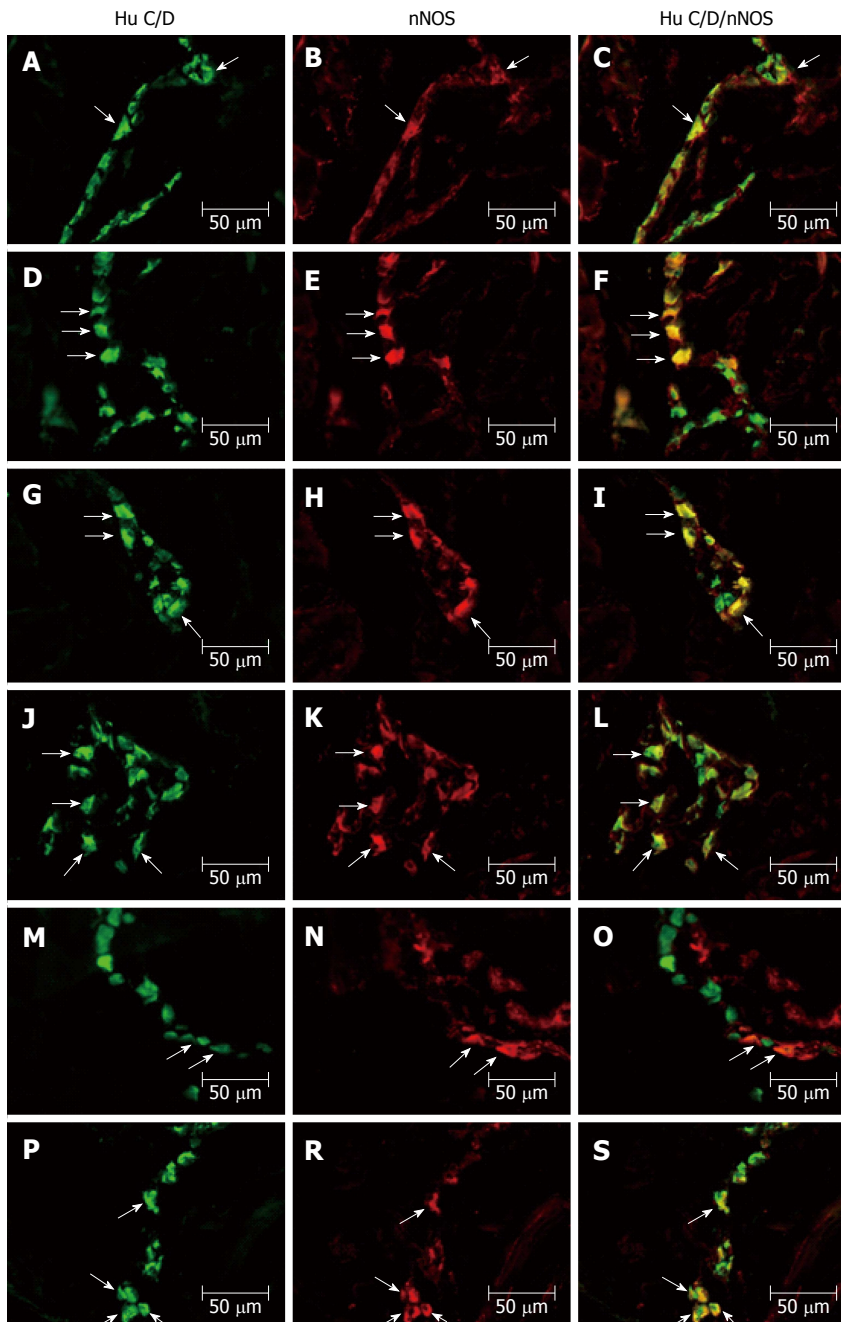


Figure 1 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to nNOS. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to nNOS; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and nNOS; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to nNOS; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and nNOS; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to nNOS; I: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and nNOS; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to nNOS; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to nNOS; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and nNOS; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to nNOS; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. nNOS: Neuronal isoform of nitric oxide synthase; Hu C/D: Pan-neuronal marker.

since spontaneous diabetes in large domestic animals such as the pig are extremely rare^[25], it is important to select an appropriate method leading to the

induction of diabetes and, thus, the development of hyperglycemia. Total pancreatectomy is a technique involving a considerable mortality rate of animals and is

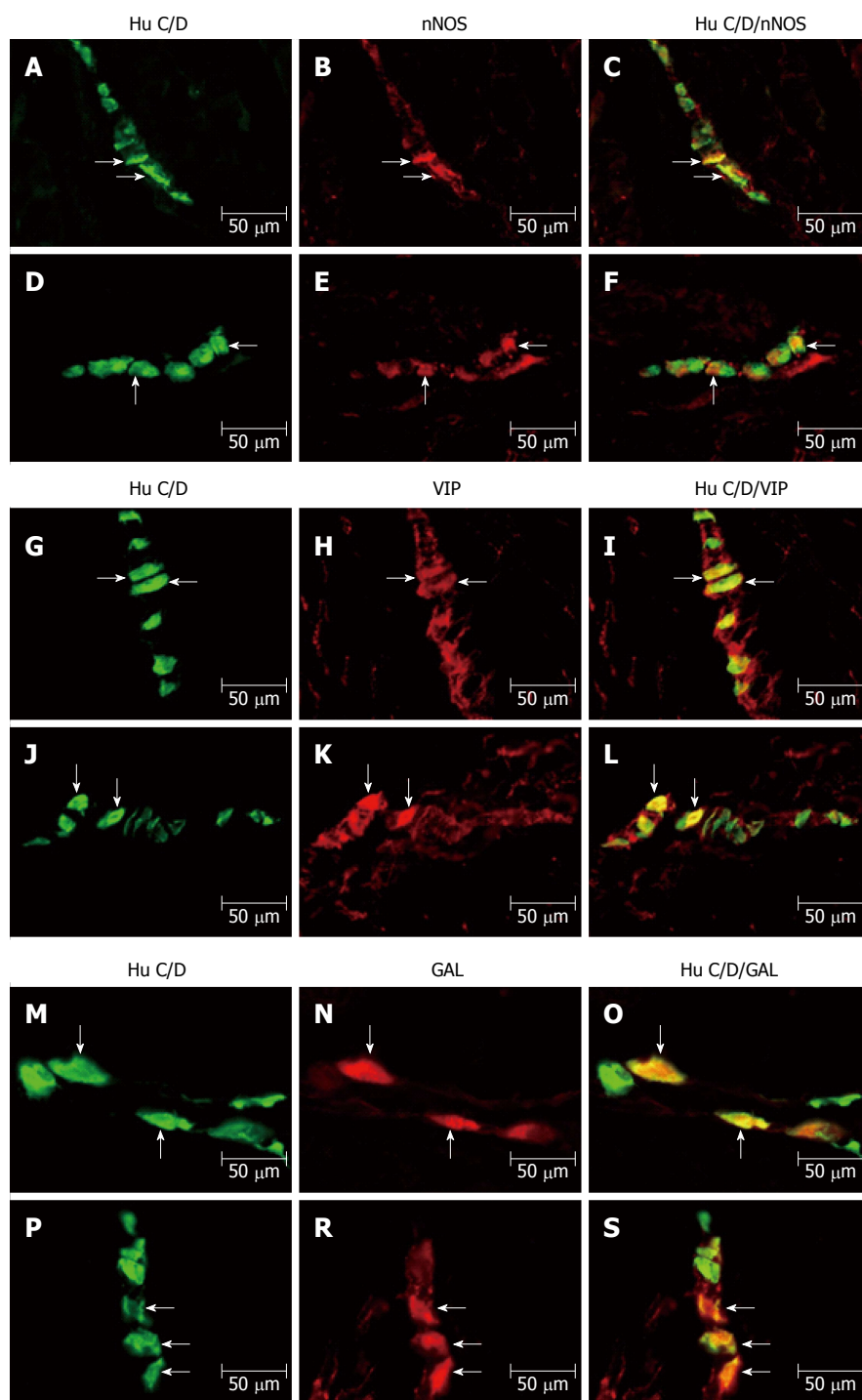


Figure 2 Submucosal ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to nNOS, VIP and GAL. A: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; B: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to nNOS; C: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and nNOS; D: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; E: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to nNOS; F: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and nNOS; G: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to VIP; I: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and VIP; J: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to VIP; L: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP; M: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; N: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and GAL; O: Submucosal ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and GAL; P: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; R: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to GAL; S: Submucosal ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. nNOS: Neuronal isoform of nitric oxide synthase; VIP: Vasoactive intestinal peptide; GAL: Galanin; Hu C/D: Pan-neuronal marker.

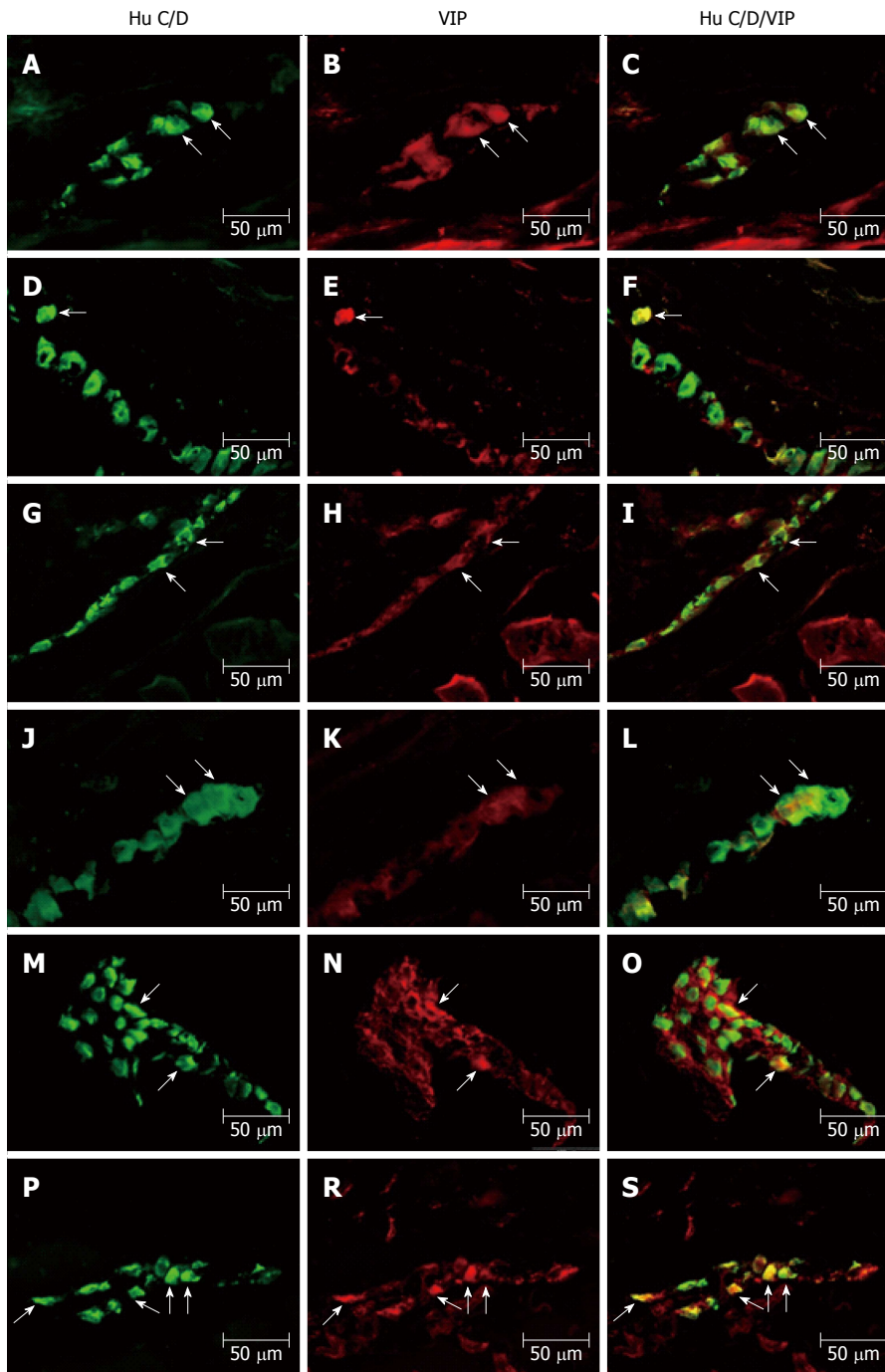


Figure 3 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to VIP. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to VIP; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and VIP; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to VIP; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and VIP; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to VIP; I: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D and VIP; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to VIP; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to VIP; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and VIP; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to VIP; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and VIP. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. VIP: Vasoactive intestinal peptide; Hu C/D: Pan-neuronal marker.

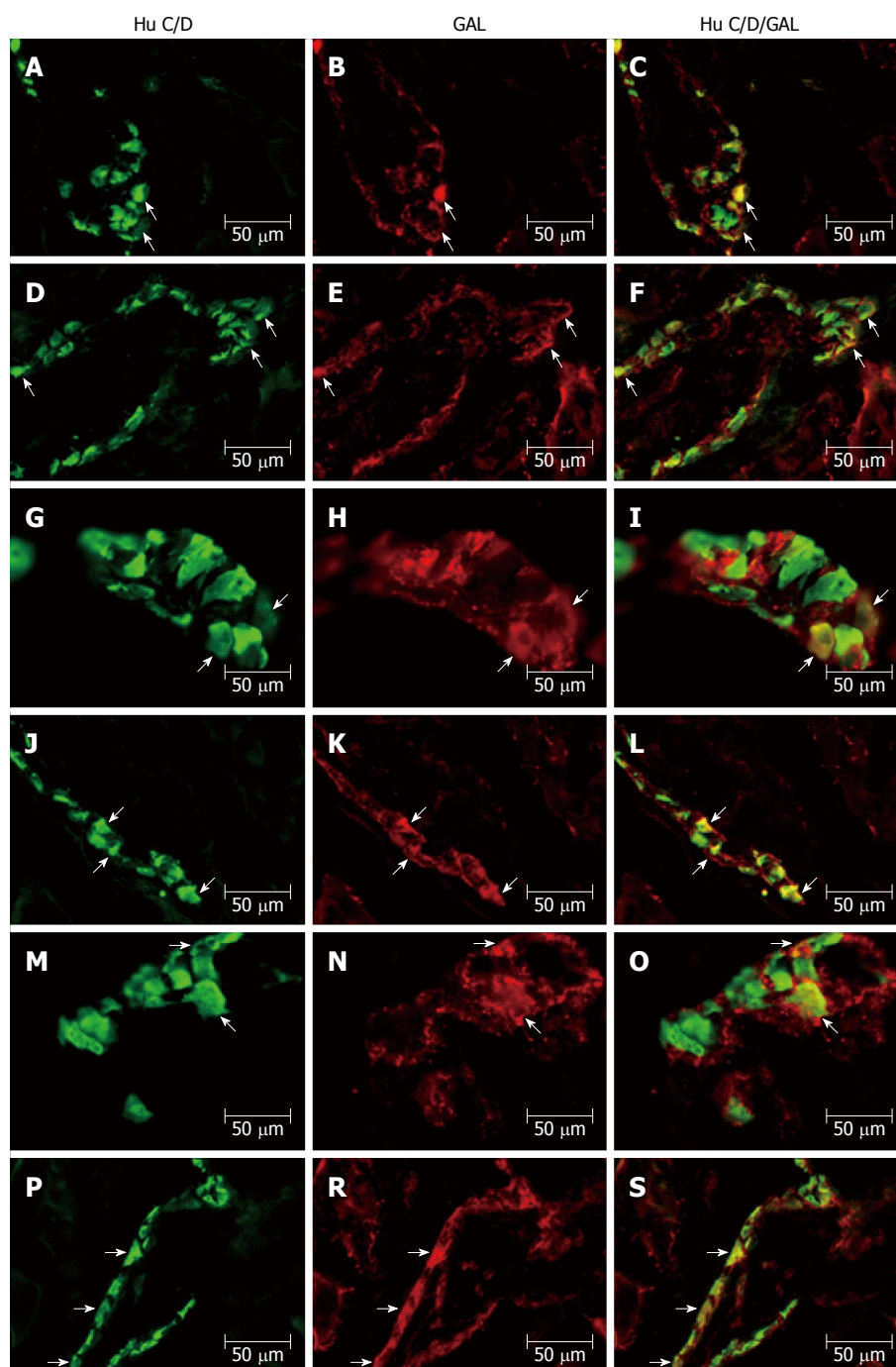


Figure 4 Myenteric ganglion of the porcine stomach under physiological condition and after streptozotocine treatment immunoreactive to GAL. A: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D; B: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to GAL; C: Myenteric ganglion of the porcine antrum under physiological condition immunoreactive to Hu C/D and GAL; D: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D; E: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to GAL; F: Myenteric ganglion of the porcine antrum after streptozotocine treatment immunoreactive to Hu C/D and GAL; G: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to Hu C/D; H: Myenteric ganglion of the porcine corpus under physiological condition immunoreactive to GAL; I: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D; J: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to GAL; K: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL; L: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL; M: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D; N: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to GAL; O: Myenteric ganglion of the porcine pylorus under physiological condition immunoreactive to Hu C/D and GAL; P: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to Hu C/D; R: Myenteric ganglion of the porcine pylorus after streptozotocine treatment immunoreactive to GAL; S: Myenteric ganglion of the porcine corpus after streptozotocine treatment immunoreactive to Hu C/D and GAL. The right column of the pictures shows the overlap of both stainings. Colocalization of both antigens in the studied cell bodies are indicated with arrows. GAL: Galanin; Hu C/D: Pan-neuronal marker.

not presently used^[25]. Therefore, in the present study we used streptozotocin administered intravenously in order to induce diabetes. This method is widely-accepted and used in many studies to determine the side-effects of elevated blood glucose^[26]. In our study, the choice of streptozotocin as a diabetogenic substance also turned out to be the opposite, because all animals developed hypoglycemia during the first week of experiment and we did not note mortality falling during the study. Moreover, it is worth underlining that the type 1 STZ-induced hyperglycemic/diabetic pig model has been predominately validated in studies of the cardiovascular complications as well as in peripheral neuropathy^[27,28]. On the other hand, diabetic gastroenteropathy, especially concerning the chemical phenotyping of enteric neurons in this model is unclear. The available data describing neurochemical changes in the ENS of hyperglycemic/diabetic animals are limited to the studies on small animal models, mainly rat and mice^[29-34].

Our results demonstrated that the porcine ENS, exhibits alterations in the chemical coding of the inhibitory neurons after six weeks of sustained hyperglycemia. It is generally recognized that NO, together with a VIP, acts as inhibitory neurotransmitter in muscle and submucosal layer of gastrointestinal tract^[14,15]. As shown in our results, a visible increase in nNOS expression was presented in the myenteric ganglion of the corpus and pylorus. Contrary to these results, in the MG of the antrum and in the SG of the corpus, changes in the synthesis of nNOS have not been noted. As mentioned above, the previous studies, of the chemical coding of enteric neurons were conducted mainly on rodents. The obtained results indicate a differential response of enteric neurons, depending on the duration time of hyperglycemia and the gastrointestinal tract region studied. Belai *et al.*^[29,30] described a decrease in nNOS expression in the antrum, while in the small intestine and colon the same authors did not find statistically important changes compared to the control group. Meanwhile, another study revealed an increased level of nNOS in the antrum during streptozotocin-induced diabetes in rats^[32]. In turn, hyperglycemia evoked a visible increase in the number of myenteric neurons in all investigated areas and a slight decrease in VIP-LI immunoreactivity in the submucosal ganglia in the corpus. As previously reported in diabetic rats, chronic hyperglycemia caused a marked increase in the immunoreactivity of VIP, in both the submucosal and myenteric ganglion. However, in the myenteric plexus, the increase was preceded by a slight decrease in the intensity of VIP immunoreactivity^[31,34]. It is worth underlining that since these results focused on the ileum, we cannot directly relate them to the stomach.

Galanin is a neurotransmitter which can play an inhibitory or excitatory role depending on the investigated area of gastrointestinal tract as well as animal species. Galanin is also a modulator regulating

the activity of other neurotransmitters, including VIP and NO. In this study, although we have shown an essential increase of galanin expression in myenteric ganglion neurons in the antrum and pylorus, in the corpus of the stomach we did not observe statistically significant changes compared to the control group. To date, an increase in galanin immunoreactivity has only been observed in the colon of non-obese diabetic mice (NOD) and the ileum of 12-wk-old diabetic rats^[31,35]. Only in obese mice with coexisting diabetes has a decrease of galanin in the colon been noted^[35]. Taking into account our results and the available data on the expression of neuroactive substances in enteric neurons (especially on inhibitory properties) the common pattern of changes is generally acceptable, i.e. inhibitory neurotransmitters such as nNOS, VIP and GAL appear to decrease in the early stage of diabetes with an increase in later stages, possibly secondary to regeneration^[7]. Our results clearly indicate that the neurotoxic effect of chronic hyperglycemia in pigs leads to neuronal regeneration, which is reflected by an up-regulation of the synthesis of NO, VIP and GAL. It is well-known that NO and VIP have strong neuroprotective activity^[36-40]. Therefore, the ENS adapts to changes in environmental conditions, increasing the synthesis of biologically active substances which may play a protective role against cellular oxidative stress as a consequence of high glucose serum levels. Moreover, we have noted down-regulation of VIP expression in the corpus of the submucosal ganglion. The different reaction of submucosal neurons probably results from another function played by neurons which leads to regulation of secretory activity^[16]. It is worth noting that since other investigated substances within the submucosal ganglion in the corpus of the stomach did not show statistically significant changes, we can conclude that these neurons have a different sensitivity to high glucose level.

Our results may partly explain the reason of gastrointestinal motility disorders observed in people with long-term diabetes. Increase of the expression of inhibitory substances may impair the function of the pyloric sphincter relaxation and the antrum contraction. Which, in turn, contributes to gastric emptying which has been observed in humans^[11].

In conclusion, this is the first report describing changes in the expression of inhibitory substances in the intramural neurons of the stomach in hyperglycemic STZ-induced diabetic pigs. Our results indicate that after six weeks of high glucose serum level expression of inhibitory substances (such as NO), vasoactive intestinal polypeptide and galanin undergo significant changes (described as chemical phenotyping), which suggests that STZ-injected pigs might serve as a good model in early studies of autonomic nerve changes under hyperglycemic conditions. Furthermore, our data provide a background for more detailed studies on the contribution of enteric neurons to the pathogenesis

of gastrointestinal disturbances as well as reference data for further, pre-clinical studies on hyperglycemia-related autonomic neuronal changes in a species more closely related to humans.

COMMENTS

Background

Diabetes mellitus is associated with several changes in gastrointestinal (GI) tract motility that lead to nausea, bloating, abdominal pain, diarrhoea and constipation. Up to 75% of patients with diabetes can experience these symptoms. The pathogenesis of altered GI functions in diabetes is multifactorial and the role of the enteric nervous system (ENS) in this respect has gained significant attention. The neuronal remodelling affects the ratio of inhibitory neurons, which in turn leads to impaired of nerve mediated muscle responses and can contribute to the motility dysfunction seen in diabetes patients.

Research frontiers

Streptozotocin is widely used to induce experimental diabetes in animals. Its application leads to blood insulin levels decrease and result in development of hyperglycaemia. Long term elevated glucose level cause diabetic neuropathy.

Innovations and breakthroughs

This is the first study utilizing pig as a model species for human diabetes induced gastrointestinal complications. The results suggest that swine due to close physiological similarity to human especially concerning gastrointestinal tract function seems to be better model for biomedical research than rodents.

Applications

The results provide evidence that acute hyperglycaemia changes properties of neuromodulators/neurotransmitters in GI. Pharmacological modulation of selected biologically active substances can be potentially new approach for treatment of the diabetes evoked gastrointestinal complications.

Terminology

Streptozotocin treatment supplies a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, streptozotocin liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the streptozotocin action B cells undergo the destruction by necrosis.

Peer-review

The authors have provided evidence that long term episodes of artificially initiated hyperglycaemia induced changes of expression of neuronal isoform of nitric oxide synthase, vasoactive intestinal peptide and galanin in inhibitory neurons inside the stomach ENS. Those changes might be responsible for broad spectrum of gastrointestinal disturbances.

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Basic Study

HOX transcript antisense intergenic RNA represses E-cadherin expression by binding to EZH2 in gastric cancer

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Abstract

AIM

To clarify the mechanisms of HOX transcript antisense intergenic RNA (HOTAIR) in gastric cancer (GC) migration and invasion.

METHODS

Quantitative real-time polymerase chain reaction (qPCR) was used to detect the expression level of HOTAIR in GC tissues. The correlation of its expression with clinicopathological features was analyzed. Area under receiver operating characteristic curve (AUC_{ROC}) was constructed to evaluate the diagnostic value of HOTAIR. Wound-healing assay and Transwell assay were performed to detect the biological effects of HOTAIR in GC cells. qPCR, western blot and

immunohistochemistry were used to evaluate the mRNA and protein expression of E-cadherin. RNA-binding protein immunoprecipitation was used for the analysis of EZH2 interactions with HOTAIR. Chromatin immunoprecipitation assay was performed to investigate direct interactions between EZH2 and E-cadherin.

RESULTS

The expression of HOTAIR was up-regulated in GC tumorous tissues compared with the para-tumorous tissues ($P < 0.001$). Its over-expression was correlated with tumor-node-metastasis (TNM) stage ($P = 0.024$), tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$), and poor prognosis ($P < 0.001$). Multivariate Cox regression analysis confirmed expression of HOTAIR as an independent predictor of overall survival ($P = 0.033$), together with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$). The AUC_{ROC} was up to 0.709 (95%CI: 0.623-0.785, $P < 0.001$). Knockdown of HOTAIR by siRNA in GC cells suppressed the migration and invasion of GC cells. Significantly negative correlation between HOTAIR and E-cadherin was found in GC tissues and cell lines, and HOTAIR contributed to the regulation of E-cadherin through binding to EZH2 with the E-cadherin promoter.

CONCLUSION

HOTAIR may play a pivotal role in tumor cell migration and invasion. It can be used as a potential diagnostic and prognostic biomarker for GC.

Key words: Long noncoding RNA; HOX transcript antisense intergenic RNA; Gastric cancer; Migration and invasion; E-cadherin

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Core tip: In this study, we found that HOX transcript antisense intergenic RNA (HOTAIR) expression was up-regulated in gastric cancer (GC) tissues. High expression of HOTAIR was associated with clinicopathological characteristics and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly inhibited the invasion and migration of GC cells. We also tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. The findings from our study will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

Chen WM, Chen WD, Jiang XM, Jia XF, Wang HM, Zhang QJ, Shu YQ, Zhao HB. HOX transcript antisense intergenic RNA represses E-cadherin expression by binding to EZH2 in gastric cancer. *World J Gastroenterol* 2017; 23(33): 6100-6110 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6100.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6100>

INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide, having high rates of both incidence and mortality^[1]. Even though remarkable progress has been made in surgical resection and chemotherapy, which are the main therapeutic approaches for GC, the prognosis continues to be dismal^[2]. The main cause of treatment failure is cancer recurrence and metastasis^[3,4].

Tumor metastasis is a complex process, in which cancer cells leave the primary tumor site and migrate to distant secondary organs. Metastatic cancer cells fractionally retain their epithelial properties and obtain mesenchymal characteristics that give them the ability to invade or distract^[5]. This process is called epithelial-to-mesenchymal transition (EMT). EMT is characterized by E-cadherin expression loss and N-cadherin and vimentin up-regulation^[6]. It is regulated by many signaling pathways, transcriptional factors and post-transcriptional factors^[7]. Post-transcriptional regulatory networks include the miRNA and long noncoding (lnc)RNA families^[8]. Emerging evidence shows that lncRNAs have an important role in regulating EMT and cancer metastasis^[5,9]. Several important lncRNAs are reported to induce EMT, including highly upregulated in liver cancer (HULC)^[10], metastasis-associated lung adenocarcinoma transcript (MALAT)-1^[11], H19^[12], and HOX transcript antisense intergenic RNA (HOTAIR)^[13].

HOTAIR is identified as an oncogene involved in many kinds of cancers, including breast cancer^[14], esophageal squamous cell carcinoma^[15], colorectal cancer^[16], and GC^[13]. HOTAIR acts mainly through the polycomb repressive complex (PRC)2 (EZH2, RbAp46, RbAp48 and SUZ12)^[17], which trimethylates histone H3 lysine-27 (H3K27) of the HOXD locus and repress HOXD gene expression^[14]. EZH2 which is the core catalytic component of the PRC2, can affect cancer progression by altering H3K27 trimethylation and silencing transcription^[17].

A previous study showed that silencing of HOTAIR inhibits GC cell migration, invasion and metastasis, and reverses the EMT in GC cells^[13]. HOTAIR in combination with PRC2 could epigenetically inhibit miR34a, and control C-Met (HGF/C-Met/Snail pathway) and Snail, thus promoting the EMT process of GC cells and tumor metastasis^[13]. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis have not yet been fully investigated.

Up-regulation of HOTAIR expression in GC tissues was found in the present study. We also found that over-expression of HOTAIR was related to the clinicopathological characteristics of and poor prognosis in GC patients. Additional experiments revealed that HOTAIR knockdown significantly repressed migration and invasion. Finally, we tested whether HOTAIR recruited EZH2 to promote tumor cell migration and invasion by repressing E-cadherin in GC. These studies

Table 1 Relationships between the expression of HOTAIR and clinicopathological characteristics in 65 patients with gastric cancer

Characteristic	Expression of HOTAIR		P value
	Low <i>n</i> = 32	High <i>n</i> = 33	
Sex			0.408
Female	7	11	
Male	25	22	
Age in yr			0.455
≤ 60	17	21	
> 60	15	12	
Histological grade			1.000
Well/moderate	14	15	
Other	18	18	
Tumor invasion depth, T			0.018
Tis, T1,T2	15	6	
T3 or above	17	27	
Lymph node metastasis, N			0.023
N0	12	4	
N1 or above	20	29	
TNM stage			0.024
I/II	23	14	
III/IV	9	19	
Tumor location			0.102
Antrum	8	15	
Cardia	10	9	
Angulus	13	5	
Body	1	3	
Full stomach	0	1	
Laure's classification			0.373
Intestinal	17	15	
Diffuse	12	17	
Mixed	3	1	

will help clarify the role of HOTAIR in GC progression and its potential as a therapeutic target.

MATERIALS AND METHODS

Tissue collection and ethics statement

A cohort of 65 primary GC patients was enrolled in this study, each of who underwent surgery at the First Affiliated Hospital of Nanjing Medical University and Jining NO.1 People's Hospital between 2008 and 2009. None received chemotherapy or radiotherapy prior to surgery. This study was approved by the University Ethics Committee. All patients participated after providing informed consent. Tumor stage was evaluated in accordance with the tumor-node-metastasis (TNM) classification system (UICC/AJCC 2002). Clinical characteristics are shown in Table 1. Patients discharged from hospital were followed up routinely and according to a scheduled program, at least once a year.

Cell lines and culture conditions

Human GC cell lines SGC-7901 and BGC-823 were obtained from the Chinese Academy of Sciences Committee on Type Culture Collection Cell Bank. Cells were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium (Gibco-BRL, Shanghai, China) supplemented with 10% fetal bovine serum (FBS)

(Invitrogen, Shanghai, China), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) in an incubator at 37 °C with 5% CO₂.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from the frozen tissues using TRIzol reagent (Invitrogen). RNA concentrations were estimated by spectrophotometer absorbance readings of 260 nm. One microgram of total RNA was used for reverse transcription to cDNA with a Reverse Transcription Kit (TaKaRa, Shiga, Japan). An ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, United States) was used to quantify HOTAIR. Ten microliters of SYBR Premix ExTaq (TaKaRa) was mixed. Primer sequences are listed in Supplementary Table 1. The relative expression was calculated using the equation: $\Delta Ct = Ct(\text{target gene}) - Ct(\text{GAPDH})$; Fold change = $2^{-[\Delta Ct(\text{tumour}) - \Delta Ct(\text{normal})]}$ [18].

Cell transfection

HOTAIR small interfering (si)RNAs and negative control siRNA (si-NC) were purchased from Invitrogen. The siRNA sequences are listed in Supplementary Table 1. BGC-823 or SGC-7901 cells were grown in 6-well plates until confluent, then transfected with Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were harvested for qPCR or western blot analysis.

Wound-healing assay

Cells were seeded in 6-well plates in normal cell growth medium and incubated to confluence. A 20-μL tip was used to make a straight scratch, simulating a wound. The medium was changed to medium containing 1% FBS. At different time points, images of the plates were acquired using a microscope.

Cell migration and invasion assays

For the migration assays, at 48 h after transfection, 5×10^4 cells in serum-free medium were placed into the upper chamber of an insert (8-μm pore size; Millipore, Bedford, MA, United States). For the invasion assays, 10^5 cells in serum-free medium were placed into the upper chamber of an insert coated with Matrigel (Sigma-Aldrich, St. Louis, MO, United States). Medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the cells remaining on the upper membrane were removed with cotton wool. Cells that had migrated or invaded through the membrane were stained with methanol and 0.1% crystal violet, imaged and counted in five random fields per well using an IX71 inverted microscope (Olympus, Tokyo, Japan). Experiments were repeated independently three times.

Western blot assay and antibodies

Cell protein lysates were separated by 15% SDS-PAGE, transferred to 0.22-μm nitrocellulose membranes

(Sigma-Aldrich) and incubated with specific antibodies. GAPDH antibody was used as a control. Autoradiograms were quantified by densitometry (Quantity One software; Bio-Rad, Hercules, CA, United States). Anti-E-cadherin was purchased from Abcam (Cambridge, United Kingdom). Anti-GAPDH was purchased from Cell Signaling Technology (Danvers, MA, United States).

RNA-binding protein immunoprecipitation assay

Binding protein immunoprecipitation assay (RIP) experiments were performed using a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Antibody for EZH2 RIP assays was from Abcam.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) assays were performed using an EZ-CHIP KIT (Millipore). EZH2 antibody was obtained from Abcam. H3 trimethyl Lys 27 antibody was from Millipore. The ChIP primer sequences are listed in Supplementary Table 1. Quantification of immunoprecipitated DNA was performed using qPCR with SYBR Green Mix (TaKaRa). ChIP data were calculated as a percentage relative to the input DNA by the equation of $2^{(\text{Input Ct} - \text{Target Ct})} \times 0.1 \times 100$.

Immunohistochemistry

The immunohistochemical analysis of E-cadherin was performed as described previously^[19]. To quantify E-cadherin protein expression, both the intensity and extent of immunoreactivity were evaluated and scored. In the present study, staining intensity was scored as follows: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The scores of the extent of immunoreactivity ranged from 0 to 3, and were determined according to the percentage of cells that showed positive staining in each microscopic field of view (0, < 25%; 1, 25%-50%; 2, 50%-75%; 3, 75%-100%). A final score ranging from 0 to 9 was achieved by multiplying the scores for intensity and extent.

Statistical analysis

SPSS version 21.0 software was used for all statistical analysis. Significance of the differences between groups was estimated by Student's *t*-test, χ^2 test, or Mann-Whitney test. Survival curves were estimated by the Kaplan-Meier method. The log-rank test was used to estimate the statistical difference between survival curves. Cox proportional hazards analysis was performed to calculate the hazard ratio (HR) and the 95% confidence interval (CI) to evaluate the association between HOTAIR expression and overall survival (OS) time. Multivariate Cox regression was performed to adjust for other covariates. Spearman correlation analysis was performed to investigate the correlation between HOTAIR and E-cadherin mRNA expression. Receiver operating characteristic (ROC)

curve was produced to evaluate the diagnostic value for differentiating between GC and adjacent non-tumor tissues. A two-tailed *P* value of ≤ 0.05 was considered statistically significant. All the graphs were plotted using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, United States).

RESULTS

HOTAIR was up-regulated in GC tissues

HOTAIR showed markedly higher expression in GC tissues as compared with matched adjacent non-tumor tissues ($P < 0.001$; Figure 1A).

Diagnostic value of using HOTAIR as a marker

An ROC curve was generated by comparing HOTAIR expression in GC tissues to expression in matched adjacent non-tumor tissues. With a cut-off value of 9.31, the area under the ROC curve (AUC_{ROC}) reached 0.709 (95%CI: 0.623-0.785, $P < 0.001$; Figure 1B). The sensitivity was 64.62%, the specificity was 75.38%, and Youden's index was 0.4.

HOTAIR expression and clinicopathological factors in GC

Patients with GC were divided into two groups based on the cut-off ratio of HOTAIR expression (2.35-fold) in tumor tissues: high-expression group ($n = 33$) and low-expression group ($n = 32$) (Figure 1C). Table 1 showed that HOTAIR over-expression was significantly correlated with tumor invasion ($P = 0.018$), lymph node metastasis ($P = 0.023$) and higher TNM stage ($P = 0.024$). However, there was no correlation between HOTAIR expression and age, sex and histological grade ($P > 0.05$).

Up-regulation of HOTAIR associated with poor survival of GC

To assess the impact of HOTAIR expression on OS of GC patients, Kaplan-Meier analysis and log rank test were used. The results revealed that patients in the high-expression group had a shorter OS (median OS: 25.9 mo) than those in the low-expression group (median OS: 42.5 mo, $P < 0.001$; Figure 1D). Univariate analyses of clinical variables considered as potential predictors of survival are shown in Table 2. Further analysis in a multivariate Cox proportional hazards model showed that HOTAIR expression ($P = 0.033$), along with TNM stage ($P = 0.002$) and lymph node metastasis ($P = 0.002$), were strongly associated with OS. These results revealed that HOTAIR expression was an independent prognostic indicator of OS.

Knockdown of HOTAIR suppressed migration and invasion of GC cells

The wound-healing assay showed that HOTAIR knockdown cells were significantly slower than control

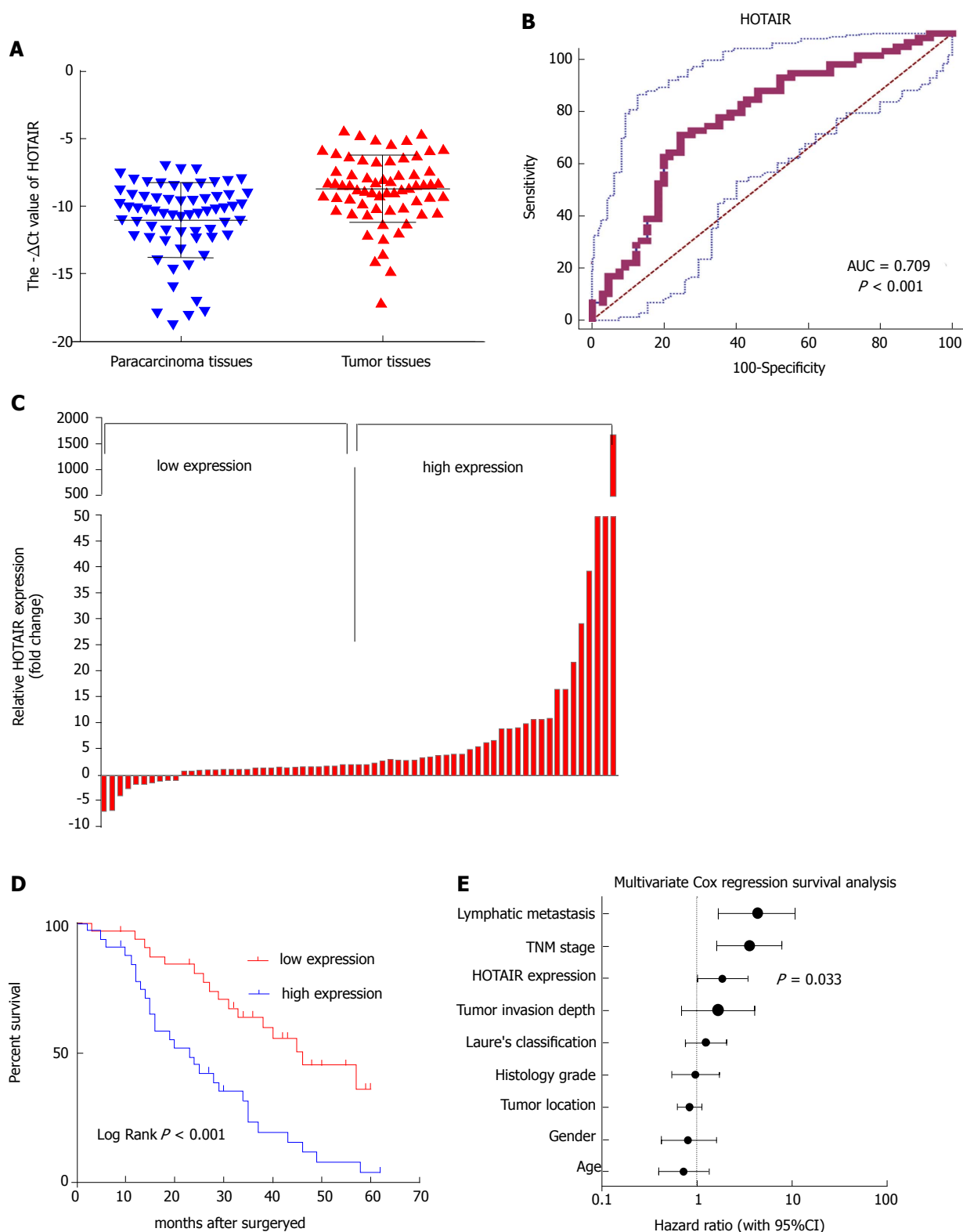


Figure 1 HOTAIR up-regulation correlates with poor survival in patients with GC. A: HOTAIR expression was significantly up-regulated in tumor tissues compared with their corresponding para-carcinoma tissues (shown as $-\Delta\text{Ct}$); B: ROC curves for observing the diagnostic value of HOTAIR; C: HOTAIR expression was classified into two groups according to HOTAIR expression levels (median split); D: Kaplan-Meier analysis of OS was analyzed according to HOTAIR expression levels; E: Different factors (including HOTAIR, tumor invasion depth, lymph node metastasis, TNM stage, histological grade, gender, tumor location, Lauren's classification and age) were analyzed for their association with patient survival using Cox regression model. The hazard ratio and 95%CI are plotted for each factor. CI: Confidence interval; GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; OS: Overall survival; ROC: Receiver operating characteristic; TNM: Tumor-node-metastasis.

cells (Figure 2A-D). Transwell assay and invasion assay demonstrated knockdown of HOTAIR notably reduced

the number of BGC-823 or SGC-7901 cells migrating across the membrane (Figure 2E-H). These results

Table 2 Univariate and multivariate analysis of clinical pathological characteristics and overall survival of 65 patients with gastric cancer

Factor	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
HOTAIR expression	2.943	1.580-5.483	0.001	1.998	1.058-3.772	0.033
Tumor invasion depth, T1, T2/above	2.651	1.316-5.341	0.006	1.676	0.691-4.067	0.254
Lymphatic metastasis, absent/present	3.470	1.459-8.250	0.005	4.324	1.701-10.993	0.002
TNM stage, I/II, III/IV	4.494	2.351-8.592	< 0.001	3.598	1.624-7.975	0.002
Histology grade, well, moderate/others	0.967	0.535-1.750	0.913			
Age, < 60/> 60	0.730	0.396-1.344	0.312			
Sex, male/female	0.822	0.428-1.581	0.557			
Tumor location	0.839	0.623-1.131	0.250			
Lauren's classification	1.256	0.757-2.084	0.378			

confirmed that knockdown of HOTAIR inhibited the migration and invasion of GC cells.

HOTAIR was inversely correlated with E-cadherin in GC tissue and cell lines

The HOTAIR and E-cadherin expression levels in GC tissues were detected by qPCR and immunohistochemistry. E-cadherin expression was significantly down-regulated in tumor tissues ($P = 0.001$), as compared with the para-tumorous tissues (Figure 3A and B). E-cadherin mRNA levels were negatively associated with HOTAIR expression in GC tissues ($r^2 = -0.298$, $P = 0.016$) (Figure 3C). HOTAIR silencing strikingly enhanced the expression of E-cadherin at transcript (Figure 3D and E) and protein (Figure 3F and G) levels in BGC-823 and SGC-7901 cells. These results demonstrated that HOTAIR promoted EMT in GC tissues and cell lines, and that HOTAIR over-expression was significantly associated with migration and invasion.

HOTAIR recruited EZH2 to inhibit the expression of E-cadherin

HOTAIR plays its role in the epigenetic regulation of gene expression mainly by binding to PRC2 complex. We firstly used RIP assay to investigate whether HOTAIR could bind to EZH2, which is the core catalytic component of the PRC2. The results showed that endogenous HOTAIR was abundant in the anti-EZH2 RIP fraction (Figure 4A), validating that HOTAIR can bind to EZH2 in GC cells. Then we conducted ChIP assay to determine whether HOTAIR regulates E-cadherin *via* recruiting EZH2 in GC cell lines. ChIP assay demonstrated that knockdown of HOTAIR significantly reduced the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4B). The rescue experiments showed that up-regulation of HOTAIR increased the binding of EZH2 and H3K27me3 with the E-cadherin promoter in GC cells (Figure 4C). These results indicated that HOTAIR inhibits E-cadherin expression partly through combining with EZH2.

nucleotides. Dysregulation of lncRNAs has been found in GC cells and their aberrant expression is correlated with tumorigenesis, metastasis, prognosis or diagnosis^[20,21].

We found that HOTAIR was markedly up-regulated in GC tissues, which was correlated with the invasion of primary tumor, lymph node metastasis, and TNM stage. The ROC curve used for distinguishing between GC tissues and normal tissues showed that HOTAIR is a promising cancer biomarker. Univariate and multivariate Cox regression analyses demonstrated that HOTAIR is a valuable prognostic factor independent of major clinicopathological features. We also found that decreased HOTAIR expression inhibited migration and invasion of tumor cells.

To clarify the molecular mechanism of HOTAIR action contributing to GC metastasis, we also investigated potential target genes involved in cell migration and invasion. Previous studies have revealed that the expression of epithelial markers (such as E-cadherin) are increased following HOTAIR knockdown in GC cells^[13,22,23]. Further mechanistic studies have shown that by recruiting and binding to PRC2, HOTAIR could epigenetically silence miR34a expression to promote GC cell EMT and metastasis^[13]. However, there are still some issues that need to be clarified. For one thing, E-cadherin is a target gene of EZH2. Because EZH2 can mediate transcriptional silencing of E-cadherin through trimethylation of H3 lysine 27^[24,25], and HOTAIR binds to EZH2^[14]. Furthermore, a significantly negative correlation was found between HOTAIR and E-cadherin in GC tissues in the present study, and knockdown of EZH2 also up-regulated E-cadherin expression in GC cells as shown by another study^[26]. So, why does HOTAIR not regulate expression of E-cadherin directly by binding to EZH2? We speculate that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin *via* EZH2 directly. To confirm this, we performed ChIP analysis in HOTAIR-silenced GC cell lines. Not unexpectedly, HOTAIR contributed to the regulation of E-cadherin *via* recruiting EZH2 and H3K27me3 to the E-cadherin promoter. Our findings provide additional insight into the mechanisms by which HOTAIR promotes GC migration and invasion.

DISCUSSION

lncRNAs are non-protein-coding transcripts > 200

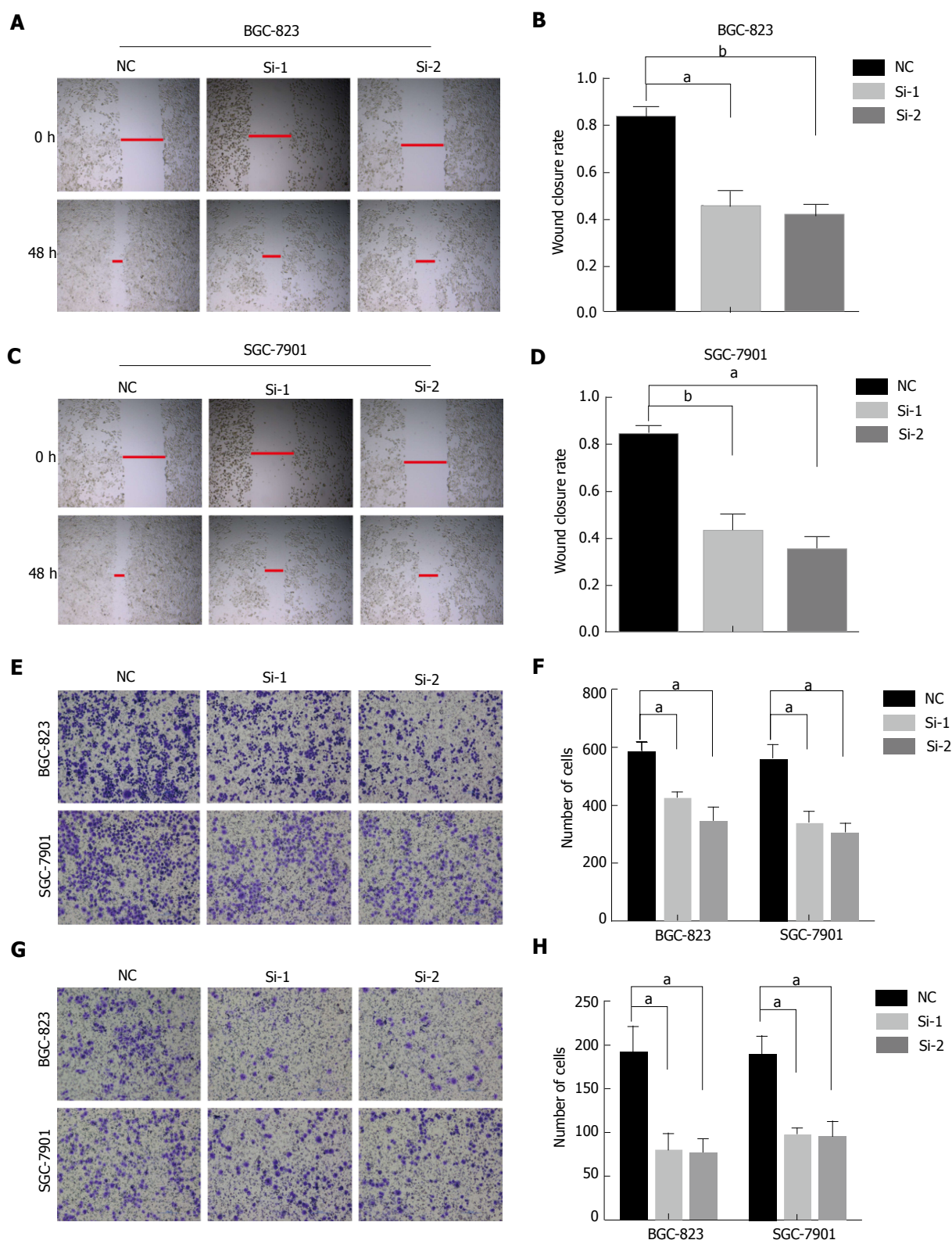


Figure 2 HOTAIR knockdown decreases migration and invasion of gastric cancer cells. A and B: HOTAIR knockdown samples exhibited slower scratch closure rate by the wound-healing detection in BGC-823 cells; C and D: Wound-healing assay in SGC-7901 cells with HOTAIR silencing; E and F: Representative pictures of cell migration across the membrane in BGC-823 and SGC-7901 cells with HOTAIR reduction; G and H: Representative pictures of Transwell invasion assay in BGC-823 and SGC-7901 cells with HOTAIR depletion. (^a $P < 0.05$, ^b $P < 0.01$). GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA.

In conclusion, we demonstrated that HOTAIR was significantly up-regulated in GC tissues, and its

overexpression was correlated with tumor progression and poor prognosis. We can distinguish between

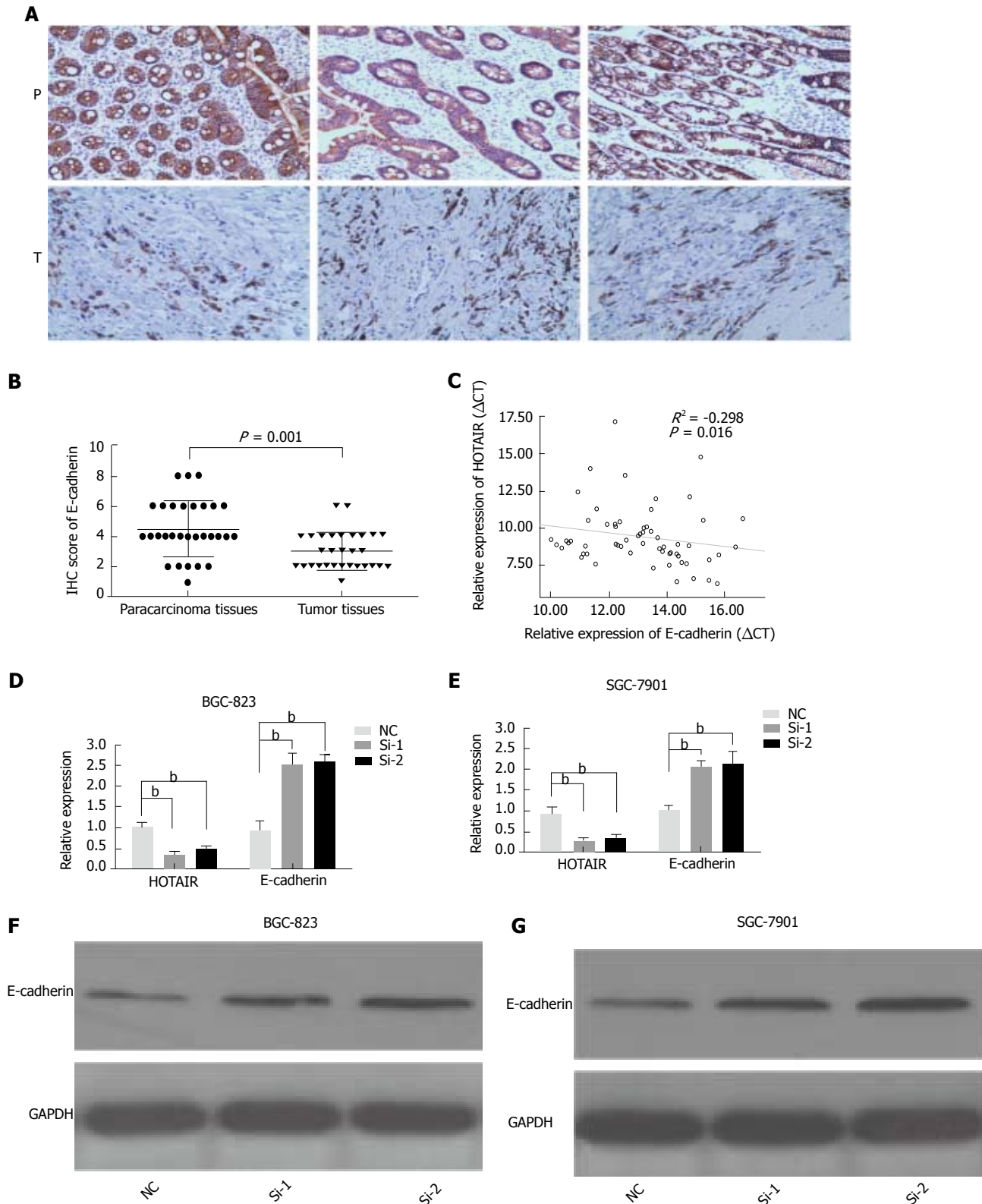


Figure 3 HOTAIR negatively correlates to E-cadherin expression in GC tissues and cell lines. **A:** E-cadherin immunostaining in para-carcinoma tissues (denoted as 'P') and GC tissues (denoted as 'T'); **B:** Representative E-cadherin protein levels and scores in GC tissues and para-carcinoma tissues was analyzed by immunohistochemistry ($n = 30$); **C:** Negative correlation between E-cadherin mRNA levels and HOTAIR levels in 65 GC samples; **D, E:** Relative expression of HOTAIR and E-cadherin in BGC-823 and SGC-7901 cells treated with siRNA for 48 h; **F, G:** Western blot analysis of E-cadherin after HOTAIR-siRNA (Si1 and Si2) treatment for 48 h in BGC-823 and SGC-7901 cells. $^aP < 0.05$, $^bP < 0.01$. GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; si: Small interfering.

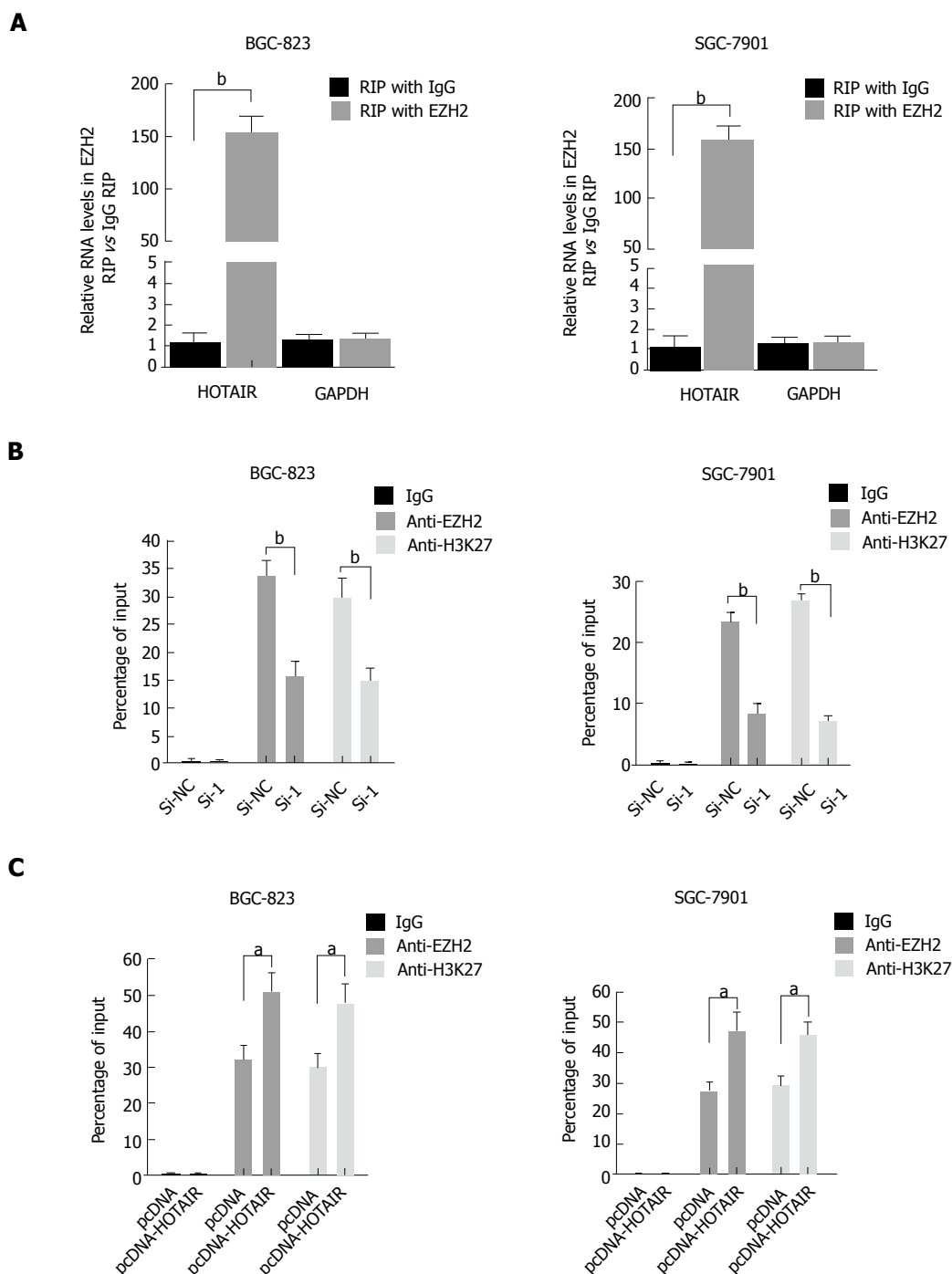


Figure 4 HOTAIR represses E-cadherin expression by associating with EZH2. A: RIP experiments were performed in BGC-823 and SGC-7901 cells, and HOTAIR levels were analyzed in co-precipitated RNA using qPCR. The fold-enrichment of HOTAIR in EZH2 RIP is relative to its matching IgG control RIP (^a $P < 0.05$, ^b $P < 0.01$); B: ChIP of EZH2 and H3K27me3 in the E-cadherin promoter regions after siRNA treatment targeting si-NC or HOTAIR-siRNA-1 in BGC-823 and SGC-7901 cells. qPCR was performed to quantify ChIP assay results. Enrichment was quantified relative to input controls. Antibody directed against IgG was used as a negative control. Results are represented as the average \pm SD based on three independent experiments. ^a $P < 0.05$, ^b $P < 0.01$; C: ChIP of EZH2 and H3K27me3 in the E-cadherin promoter regions after transfected with empty vector (denoted as 'pcDNA') or pcDNA-HOTAIR in BGC-823 and SGC-7901 cells. qPCR was performed to quantify ChIP assay results. Enrichment was quantified relative to input controls. Results are represented as the average \pm SD based on three independent experiments (^a $P < 0.05$, ^b $P < 0.01$). ChIP: Chromatin immunoprecipitation; GC: Gastric cancer; HOTAIR: HOX transcript antisense intergenic RNA; qPCR: Quantitative polymerase chain reaction; RIP: RNA-binding protein immunoprecipitation; SD: Standard deviation; si: Small interfering.

cancerous and non-cancerous lesions based on the expression of HOTAIR. HOTAIR may regulate the invasive ability of GC cells, partially *via* EMT regulation. Our findings have provided new insight into the GC pathogenesis, which benefits diagnosis and therapy in cancer.

COMMENTS

Background

Gastric cancer (GC) is one of the most aggressive malignancies with high morbidity and mortality worldwide. Treatment options are limited due to the lack of knowledge of the molecular and genetic bases of gastric carcinogenesis. A deeper understanding of the molecular mechanisms of GC will shed light on its

pathogenesis, and identification of new biomarkers for diagnosis and prognosis may improve individualized treatment strategies in the future.

Research frontiers

Emerging evidence suggest that long non-coding (lnc)RNA may play a role in gastric carcinogenesis. HOX transcript antisense intergenic RNA (HOTAIR) is one of the well-documented lncRNAs, which is aberrantly expressed in several tumors and plays a crucial role in cancer development. However, the overall clinical role of HOTAIR in GC and the molecular mechanisms of HOTAIR involved in GC cell metastasis has not yet been well investigated.

Innovations and breakthrough

The authors demonstrated that HOTAIR promotes tumor cell migration and invasion by repressing E-cadherin via EZH2 directly. Their findings have provided additional insight into the mechanisms of gastric carcinogenesis.

Applications

The results from this study provide novel clues for further investigation of HOTAIR as a potential biomarker and therapeutic target for GC.

Terminology

RNA-binding protein immunoprecipitation assay was used for the analysis of EZH2 interactions with HOTAIR. ChIP assay was performed to investigate direct interactions between EZH2 and E-cadherin.

Peer-review

The study aimed to investigate the clinical significance and the mechanism behind lncRNA HOTAIR in GC. The overall study is solid and well designed. The results are consistent with the proposed hypothesis.

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Basic Study

Ca²⁺/calmodulin-dependent protein kinase II regulates colon cancer proliferation and migration *via* ERK1/2 and p38 pathways

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Abstract

AIM

To investigate the role of calmodulin-dependent protein kinase II (CaMK II) in colon cancer growth, migration and invasion.

METHODS

CaMK II expression in colon cancer and paracancerous tissues was evaluated *via* immunohistochemistry. Transcriptional and posttranscriptional levels of CaMK II in tissue samples and MMP2, MMP9 and TIMP-1 expression in the human colon cancer cell line HCT116 were assessed by qRT-PCR and western blot. Cell proliferation was detected with the MTT assay. Cancer cell migration and invasion were investigated with the Transwell culture system and wound-healing assay.

RESULTS

We first demonstrated that CaMK II was over-expressed in human colon cancers and was associated with cancer differentiation. In the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. Furthermore, KN93 also significantly

prohibited HCT116 cell migration and invasion. The specific inhibition of ERK1/2 or p38 decreased the proliferation and migration of colon cancer cells.

CONCLUSION

Our findings highlight CaMK II as a potential critical mediator in human colon tumor development and metastasis.

Key words: Ca²⁺/calmodulin-dependent protein kinase II; Colon cancer; Proliferation; Migration

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Core tip: In the present study, we demonstrated that calmodulin-dependent protein kinase II (CaMK II) was over-expressed in human colon cancers and was associated with cancer differentiation. We investigated the role of CaMKII in human colon cancer proliferation and migration. The results revealed that in the human colon cancer cell line HCT116, the CaMK II-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. KN93 also significantly prohibited colon cancer cell migration and invasion. Additionally, we found that ERK1/2 and p38 were the targets of CaMK II regulation. These findings highlight CaMK II as a potential critical mediator in human colon tumor development and metastasis.

Chen W, An P, Quan XJ, Zhang J, Zhou ZY, Zou LP, Luo HS. Ca²⁺/calmodulin-dependent protein kinase II regulates colon cancer proliferation and migration via ERK1/2 and p38 pathways. *World J Gastroenterol* 2017; 23(33): 6111-6118 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6111.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6111>

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer death globally. Researchers estimate that roughly 1.4 million new cases of CRC will be diagnosed and 694000 deaths will result from CRC per year^[1]. Despite improvements in multimodal anticancer strategies, the prognosis of advanced CRC is still poor, with 5-year survival rates for stage III and stage IV colon cancer at 65.4% and 12.8%, respectively^[2]. A detailed understanding of the biological processes that modulate the progression and metastasis of CRC may provide new molecular pathogenesis targets for CRC diagnosis and benefit anti-tumor therapy.

The calcium ion (Ca²⁺) is a ubiquitous intracellular signal responsible for a broad range of cellular events, such as cell growth, cytoskeletal organization, regulation of synaptic transmission, and Ca²⁺ homeostasis^[3]. The Ca²⁺/calmodulin (CaM)-dependent protein kinases (CaMK I, CaMK II and CaMKIV) are

multifunctional serine/threonine kinases whose activity is activated upon Ca²⁺/CaM binding^[4]. The upstream CaMK kinases (CaMKKs) phosphorylate a critical Thr 200 in the activation-loop to activate CaMKIV, whereas CaMKII is fully activated by the autophosphorylation of its own Thr 286^[5]. Studies have revealed that the calcium signal is involved in cancer cell proliferation, invasion, tumor growth and metastasis^[6-9]. However, fewer studies have focused on CaMKII, one of the most important sensors and regulators of the Ca²⁺ signal, in digestive cancers, especially in CRC.

In the present study, we first demonstrated that CaMKII was over-expressed in human colon cancers and was associated with cancer differentiation. We further sought to investigate the role of CaMKII in human colon cancer proliferation and migration. The results revealed that in the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. KN93 also significantly prohibits HCT116 cell migration and invasion. Additionally, we determined that CaMKII inhibition decreased the phosphorylation of ERK1/2 and p38. The specific inhibition of ERK1/2 or p38 decreased the proliferation and migration of colon cancer cells. These findings highlight CaMKII as a potential critical mediator in human colon tumor development and metastasis.

MATERIALS AND METHODS

Reagents

Antibodies against CaMKIIα, ERK1/2, phospho (p)-ERK1/2, p38, p-p38, MMP2, MMP9, TIMP-1 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). KN-93 (2-[N-(2-Hydroxyethyl)]-N-(4-methoxybenzene-sulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), KN-92 (2-[N-(4-Methoxybenzenesulfonyl)] amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), PD98059 and SB203580 were obtained from Calbiochem (La Jolla, CA, United States). Other chemicals of the highest purity were purchased from Sigma (St. Louis, MO, United States).

Tissue samples

Colon tissue samples were obtained from the Department of Gastrointestinal Surgery at Renmin Hospital of Wuhan University in accordance with local ethics committees. All samples were obtained from the primary colon sites of pretreatment cases of paracancerous lesions (*n* = 5), well-differentiated colon cancer tissues (*n* = 6) and poorly differentiated colon cancer tissues (*n* = 6). All samples were analyzed and assessed by two histological specialists working blindly.

Cell culture and treatment

The human colon cancer cell line HCT116 was obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured

in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Rockville, MD, United States) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Life Technologies, Rockville, MD, United States) at 37 °C in a humidified atmosphere with 5% CO₂. For conditioned media, 3 × 10⁵ HCT116 cells were seeded in 6-well plates and then stimulated for the indicated amount of time.

Immunostaining

Immunohistochemistry (IHC) for CaMKII was performed on paraffin-embedded tissues from paracancerous tissues, well-differentiated colon cancers and poorly differentiated colon cancers. Specimens were fixed in a 4% formaldehyde solution for 12-48 h and embedded in paraffin. IHC was performed on 3.5 µm tissue sections on slides. Heat-induced epitope retrieval was carried out. For immunofluorescent staining for CaMKII, all slides were blocked with serum solution and incubated with the primary antibody overnight at 4 °C. After rinsing again, the slides were incubated for 45 min at room temperature with secondary antibodies. Visualization was achieved with the DAB (3,3'-diaminobenzidine) detection kit, and the cell nuclei were counterstained using hematoxylin. After staining, five fields (at a magnification of × 100) were randomly selected in each slide. Staining was quantified with Image J software and evaluated by two investigators following the "blinded" principle.

MTT assay

Cell proliferation was measured with the MTT assay (ATCC, Manassas, VA, United States). A total of 5 × 10³ cells were seeded at the well bottoms of 96-well culture plates and treated with TGFβ1 or conditioned medium from fibroblasts or colon cancer cells in triplicate. After treatment, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] was added to the culture medium (0.5 mmol/L), and plates were incubated for 2 h at 37 °C with 5% CO₂. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 540 nm with a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, United States).

Western blot analysis

Protein from unstained paraffin-embedded samples was deparaffinized and rehydrated *via* successive washes in xylene/ethanol and water. After incubation in extraction buffer at 100 °C for 20 min followed by a 2 h incubation at 80 °C, proteins were obtained by centrifugation. Whole cell lysates were prepared with RIPA lysis buffer containing protease and phosphatase inhibitor cocktail. Protein concentrations of cell lysates were determined with the bicinchoninic acid protein assay. A total of 30-40 µg of lysate were loaded onto a 10% SDS-PAGE gel and subjected to gel electrophoresis. Resolved proteins were transferred to a polyvinylidene fluoride membrane. Membranes

were then blocked in 5% non-fat dry milk in Tris-buffered saline with Tween (TBS-T) for 1 h and then incubated with antibodies to GAPDH, CaMKIIα (Cell Signaling Technology), ERK1/2, phospho-ERK1/2, p38, p-p38, MMP2, MMP9 and TIMP-1 at 4 °C overnight. Membranes were washed with TBS-T and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The blotting bands were visualized with enhanced chemiluminescence. GAPDH was used as a loading control.

Quantitative real-time PCR

Cells were seeded at 10⁶ cells per 10 cm dish and were allowed to grow to 80% confluency in complete media. Cells were removed with 0.025% ethylenediaminetetraacetic acid and centrifuged for 5 min at 1100 rpm. Cell pellets were resuspended in 1 mL of TRIzol (Life Technologies, Carlsbad, CA, United States), and RNA was extracted according to the manufacturer's protocol. Total RNA in unstained paraffin-embedded samples was extracted with the RNeasy FFPE Kit (Qiagen, Hilden, Germany). The SuperScript First-Strand Kit (Life Technologies) was used to synthesize cDNA from 5 µg of total RNA. Quantitative PCR was established with the RT2 SYBR Green Fluor FAST Mastermix (Qiagen, Venlo, the Netherlands) and run on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad). Data were analyzed with Bio-Rad CFX Manager 3.0 software and are shown as relative fold-changes.

For all PCR reactions, GAPDH was used as an endogenous control, and CT values were normalized to levels of GAPDH expression. Primers were designed with Integrated DNA Technologies PrimerQuest software (Coralville, IA, United States). Sequences used to analyze RNA expression include the following: CaMKII, Forward: 5'-GAG AGC ACC AAC ACC ACC ATC G-3' and Reverse: 5'-AGG CTG ACT CGT CGC CCA TCA GG-3'; MMP2, Forward: 5'-TCT CCT GAC ATT GAC CTT GGC-3' and Reverse: 5'-CAA GGT GCT GGC TGA GTA GAT C-3'; MMP9, Forward: 5'-TTG ACA GCG ACA AGA AGT GG-3' and Reverse: 5'-GCC ATT CAC GTC GTC CTT AT-3'; TIMP-1, Forward: 5'-CTT CTG GCA TCC TGT TGT TG-3' and Reverse: 5'-GGT ATA AGG TGG TCT GGT TG-3'; GAPDH, Forward: 5'-ACA GTC CAT GCC ATC ACT GCC-3' and Reverse: 5'-GCC TGC TTC ACC ACC TTC TTG-3'. The results are averaged from three independent experiments.

Migration and invasion assay

A 24-well Transwell plate (Fisher Scientific, Hampton, NH, United States) was used to measure migratory and invasive ability *in vitro*. A total of 2 × 10⁵ HCT-116 cells were plated on the bottom of each well. For the migration assay, 1 × 10⁵ colon cancer cells were plated in the top chamber with a non-coated membrane. For the invasion assay, 2 × 10⁵ HCT-116 cells were seeded in the top chamber coated with Matrigel (BD Biosciences, San Jose, CA, United States). In both

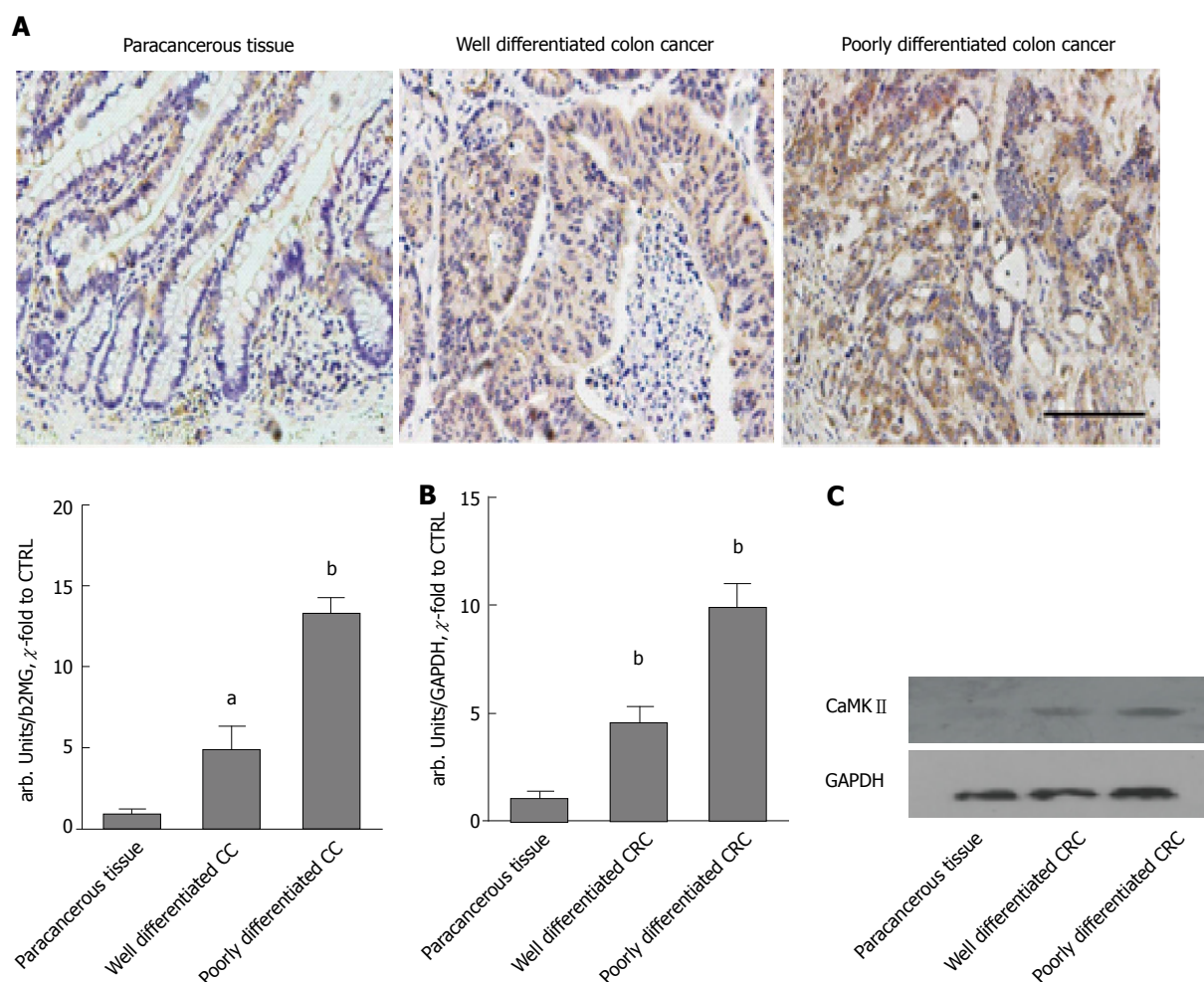


Figure 1 CaMKII over-expressed in colon cancer. A: Immunohistochemistry staining of CaMKII in paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples. Scale bar, 100 μ m; B: qRT-PCR of CaMKII mRNA in paraffin-embedded paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples; C: Western blot of CaMKII protein in paraffin-embedded paracancerous tissue, well-differentiated colon cancer and poorly differentiated colon cancer samples. All data are presented as the mean \pm standard error of at least three independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs paracancerous tissue.

assays, cells were cultured in DMEM containing 10% FBS with the indicated treatment. After incubation at 37 °C for 24 h, migrated cells were fixed and stained with the Diff-Quik stain and counted in four random fields. The experiments were performed in triplicate wells, and each experiment was performed at least two or three times as indicated.

Wound-healing assay

A wound-healing assay was performed with 6-well plates. HCT116 cells were seeded at confluency 24 h before the experiment. The cell layers were carefully scratched with 200-mL sterile pipette tips and washed twice with fresh medium. The cells were photographed under a light microscope at $\times 200$ magnification 24 h later.

Statistical analysis

All experiments were performed in triplicate. Data are expressed as the mean \pm SD. Differences between

groups were calculated *via* one-way analysis of variance. Differences were considered significant at $P < 0.05$.

RESULTS

CaMKII is over-expressed in colon cancers

The expression of CaMKII was evaluated by IHC with a monoclonal CaMKII antibody on sections obtained from formalin-fixed, paraffin-embedded samples of paracancerous tissue, well-differentiated colon cancer samples and poorly differentiated colon cancer samples. As expected, CaMKII was significantly overexpressed in the poorly differentiated colon cancer samples and well-differentiated cancer samples compared to the paracancerous tissues (Figure 1A). A quantification assay suggested that the expression of CaMKII was highest in poorly differentiated colon cancer tissues. We further evaluated CaMKII protein and mRNA levels in cancer and paracancerous

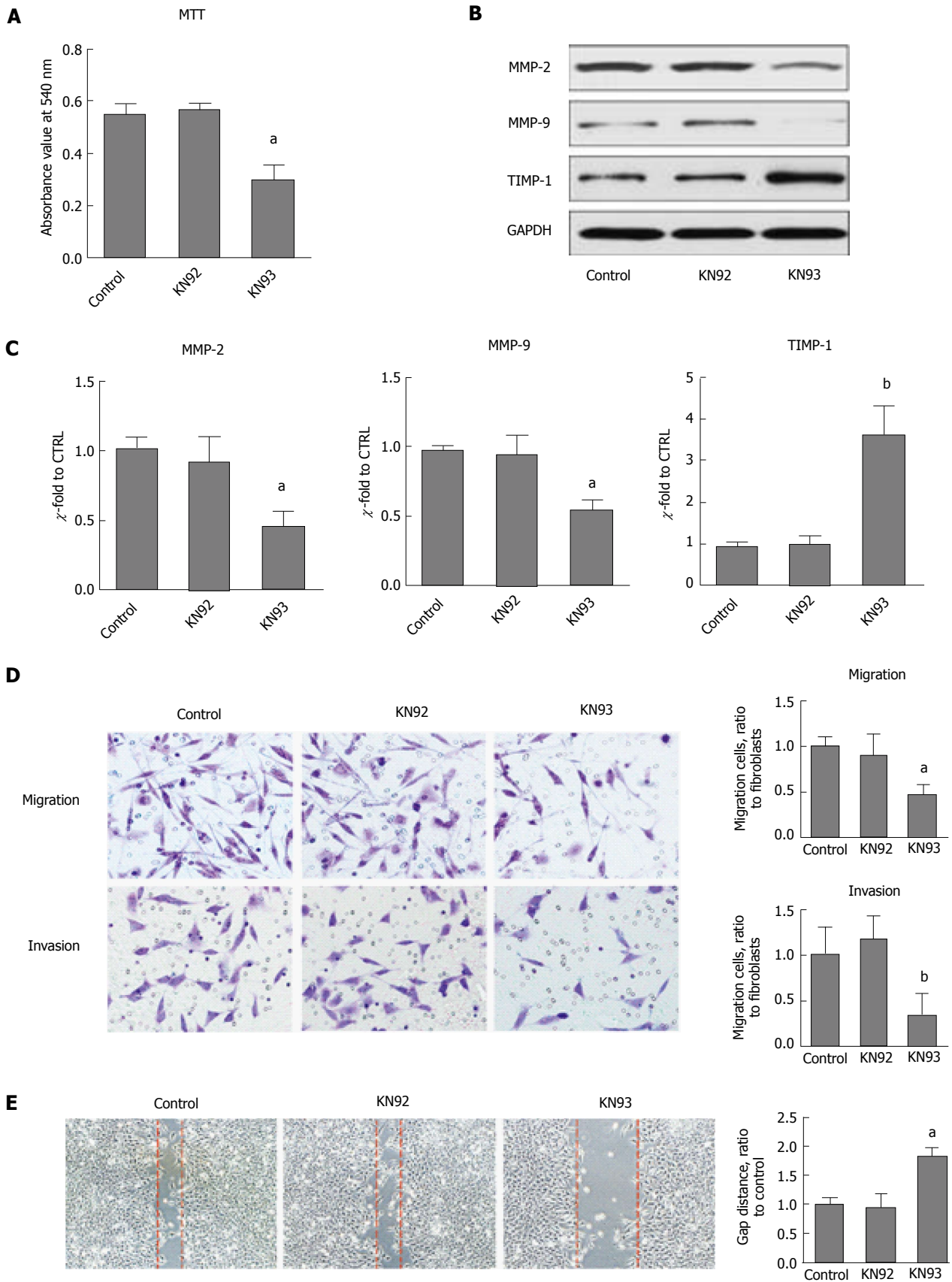


Figure 2 CaMKII is required for colon cancer proliferation and migration. Human colon cancer cells HCT116 were treated with or without KN93 or KN92 at 10 μ mol/L for 24 h. A: MTT analysis for cell proliferation; B: Western blot of MMP2, MMP9 and TIMP-1; C: qRT-PCR assay of MMP2, MMP9 and TIMP-1; D: Immigration and invasion analysis by T transwell assay; E: Wound-healing test. All data are presented as the mean \pm standard error SE of at least three independent experiments. ^a $P < 0.05$, ^b $P < 0.01$ vs control group.

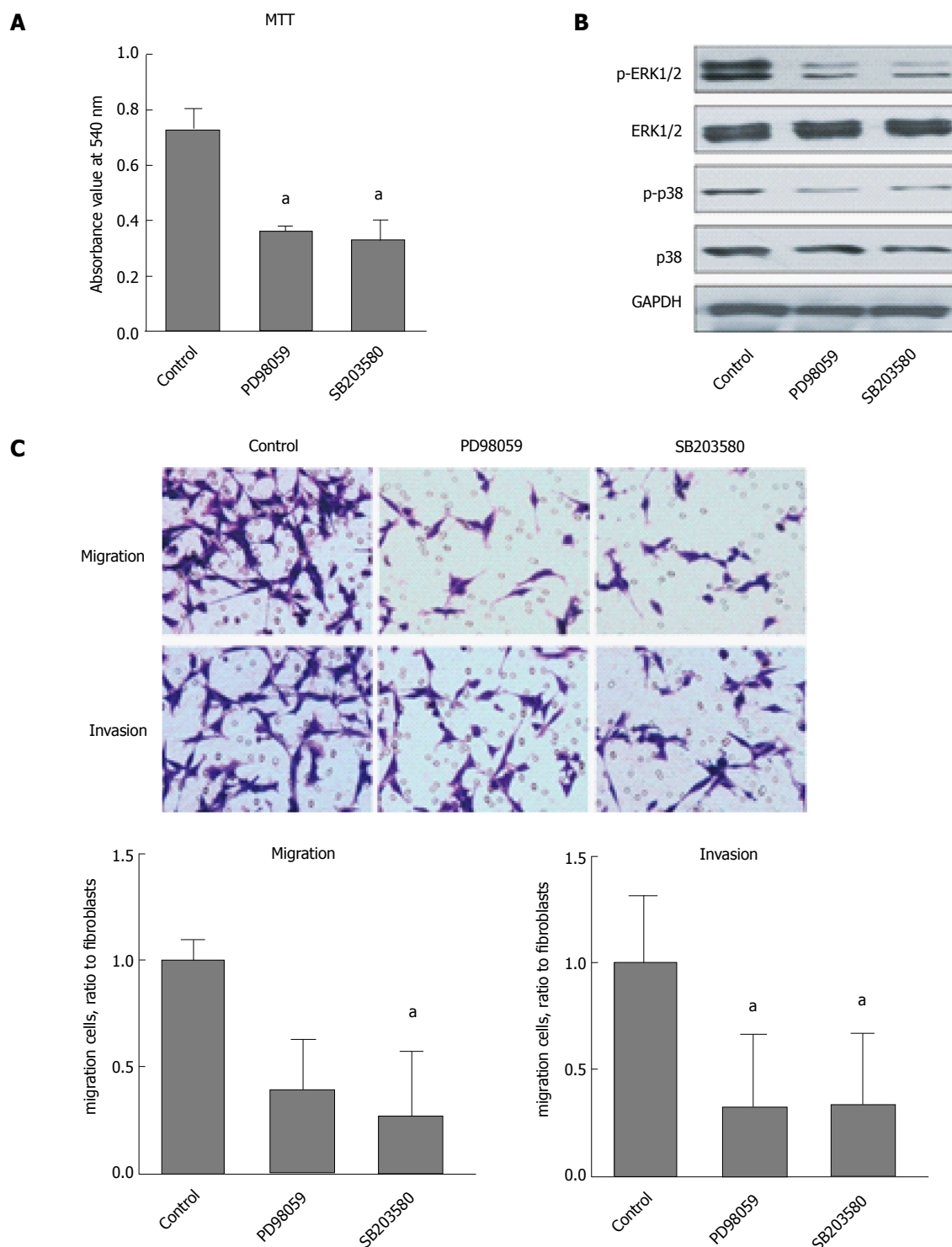


Figure 3 CaMKII regulates colon cancer cells proliferation and migration via ERK1/2- and p38-dependent pathways. Human colon cancer cells HCT116 were treated with or without PD98059 (50 $\mu\text{mol/L}$) or SB203580 (10 $\mu\text{mol/L}$) for 24 h. A: MTT assay for cell proliferation; B: Western blot analysis of ERK1/2, phosphor-ERK1/2, p38 and phosphor-p38; C: Immigration and invasion analysis by T transwell assay. All data are presented as the mean \pm standard error SE of at least three independent experiments. ^a $P < 0.05$, vs control group.

tissues. The results were similar to the findings of the above studies (Figure 1B and C). We deduced that CaMKII may be a potential regulator in colon cancer development.

CaMKII is required for colon cancer proliferation and migration

CaMKII has been shown to drive cancer growth and metastasis. To ascertain whether CaMKII regulates

colon cancer proliferation and migration, the CaMKII specific inhibitor KN93 was selected. Human colon cancer HCT116 cells were treated with KN93, and its inactive analogue, KN92, was used as an additional control. We found that KN93 inhibited colon cancer cell proliferation (Figure 2A). Importantly, in migration and invasion experiments, the motility of HCT116 cells was significantly attenuated (Figure 2B and C). Furthermore, CaMKII inhibition decreased MMP2 and MMP9 expression at both transcriptional and posttranscriptional levels. The opposite results were detected for TIMP-1 expression (Figure 2D and E). Thus, we concluded that CaMKII is required for colon cancer cell proliferation and migration.

CaMKII regulates colon cancer cell proliferation and migration via ERK1/2 and p38-dependent pathways

We further investigated the molecular mechanism of CaMKII regulation in human colon cancer cells. As shown in Figure 3, KN93, but not KN92, decreased the phosphorylation of ERK1/2 and p38, which are involved in colon cancer development. Pretreatment with specific inhibitors of ERK1/2 or p38 (PD98059, SB203580) decreased HCT116 cell proliferation, migration and invasion. These data demonstrated that the effects of CaMKII on human colon cancer are ERK1/2- and p38-dependent.

Taken together, these results indicate that CaMKII is an important regulator of human colon cancer growth and migration.

DISCUSSION

It is widely accepted that intracellular Ca^{2+} signaling pathways regulate various biological events, including cell proliferation, motility, activation and differentiation, during cancer development^[10,11]. In the present study, we demonstrated that CaMKII is over-expressed in colon cancers and highly expressed in poorly differentiated colon tumors. We highlight the role of CaMKII in colon cancer growth and metastasis.

CaMKII inhibitors can inhibit CaMKII activity by interacting with the Ca^{2+} /CaM-binding site or interfering with its catalytic activities. Previous studies revealed that CaMKII-specific inhibitors such as KN-62, KN-93, and autocamtide 2-related inhibitory peptide inhibit CaMKII-dependent processes in tumor and normal cells, causing cell cycle arrest, cellular apoptosis, or the inhibition of cell proliferation^[12-14].

Some studies have implicated CaMKII as an important player in cancer cell proliferation. CaMKII inhibition decreased ovarian cancer cell motility and decreased tumor growth and metastasis^[15]. The *in vivo* administration of KN-93 to mice xenografted with human osteosarcoma cells significantly decreased intratibial and subcutaneous tumor growth^[16]. In CRC, we demonstrated that CaMKII inhibition strongly

inhibited human colon cancer cell proliferation (Figure 2A).

Recent studies support the important role of CaMKII in cancer invasion and metastasis. The up-regulation of CaMKII α was found in primary osteosarcoma tissues from patients and in aggressive osteosarcoma cell lines^[17]. CaMKII α depletion decreased motility and invasion, whereas CaMKII α over-expression increased the tumorigenic properties of osteosarcoma cells *in vitro*. Our current results revealed the important role of CaMKII in colon cancer migration and invasion. KN93 dramatically decreased the expression of MMP2 and MMP9, increased TIMP-1 levels and prohibited the invasive motility of colon cancer cells (Figure 2). CaMKII is a critical regulator of colon cancer progression.

The MAPK pathway is one of the important downstream targets in CaMKII regulation^[4]. CaMKII directly or indirectly up-regulated multiple signaling pathways, such as ERK1/2, AKT1 and β -catenin, and is involved in regulating the survival and proliferation of non-small cell lung cancer cells^[6]. We further investigated whether ERK1/2 and p38 were involved in CaMKII pathways. Our data showed that the inhibition of CaMKII by KN93 significantly decreased ERK1/2 and p38 phosphorylation (Figure 3). The specific inhibition of ERK1/2 or p38 decreased the proliferation, migration and invasion of HCT116 cells. Therefore, CaMKII mediates colon cancer growth and migration via ERK1/2- and p38-dependent pathways.

Taken together, the current study provided evidence for CaMKII as a pivotal regulator of colon cancer cell proliferation and migration. CaMKII is a potentially important target in CRC treatment.

COMMENTS

Background

Ca^{2+} /calmodulin (CaM)-dependent protein kinase II (CaMKII) is one of the most important sensors and regulators of the Ca^{2+} signal which is involved in cancer cell proliferation, invasion, tumor growth and metastasis. Despite the critical role of CaMKII, fewer studies focus on it in digestive cancers, especially in colorectal cancer.

Research frontiers

Recently, studies have revealed CaMKII is an effective controller in ovarian cancer and osteosarcoma cell motility, cell growth and metastasis.

Innovations and breakthroughs

In the present study, the authors demonstrated that CaMKII was over-expressed in human colon cancers and was associated with cancer differentiation. Meanwhile, in the human colon cancer cell line HCT116, the CaMKII-specific inhibitor KN93, but not its inactive analogue KN92, decreased cancer cell proliferation. CaMKII inhibition also significantly prohibited colon cancer cell migration and invasion. Additionally, ERK1/2 and p38 were the targets of CaMKII regulation.

Applications

These findings highlight CaMKII as a potential critical mediator in human colon tumor development and metastasis. Its relationship to colon cancer

differentiation indicates CaMKII a potential target for clinical diagnosis and prognosis.

Terminology

CaMKII is a ubiquitous serine/threonine protein kinase. Autophosphorylation of CaMKII at threonine 286 switches the kinase from Ca^{2+} -dependent to Ca^{2+} -independent activity and leads to sustained CaMKII auto-activation. More importantly, this activation is thought to phosphorylate numerous different proteins and regulate diverse signaling pathways.

Peer-review

This is a brief study concerning the role of Ca^{2+} /calmodulin-dependent protein kinase II involved in invasion and migration of the colon cancer. The study is interesting and topical. The experiments were well designed. The manuscript is clear and structured very well.

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Basic Study

Aberrant DNA-PKcs and ERGIC1 expression may be involved in initiation of gastric cancer

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Abstract

AIM

To investigate the molecular mechanisms of gastric carcinogenesis.

METHODS

We used label-free quantification technology integrated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify differentially expressed proteins in 160 specimens of normal gastric mucosa, gastric mucosa with mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal gastric cancer (GC) collected at the Second Hospital of Lanzhou University from 2010 to 2015. Immunohistochemistry was used to verify the differentially expressed proteins detected by LC-MS/MS.

RESULTS

With a threshold of a 1.2-fold change and a *P*-value

< 0.05 between mild dysplasia, moderate dysplasia, severe dysplasia or early mucosal GC and matched normal gastric mucosa tissues, proteomic analysis identified 365 significantly differentially expressed proteins. ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by immunohistochemistry were consistent with the LC-MS/MS results.

CONCLUSION

The results suggest that aberrant ERGIC1 and DNA-PKcs expression may be involved in GC initiation.

Key words: DNA-PKcs; ERGIC1; Dysplasia; Proteomics; Gastric cancer

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Core tip: Using label-free combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), the expression of 365 proteins based on the tendency of fold change was revealed to be statistically different between the various stages of gastric cancer (GC) initiation. Furthermore, we observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by immunohistochemistry were consistent with the LC-MS/MS results. These data indicate that abnormal ERGIC1 and DNA-PKcs expression may play an important role in GC initiation.

Wang FR, Wei YC, Han ZJ, He WT, Guan XY, Chen H, Li YM. Aberrant DNA-PKcs and ERGIC1 expression may be involved in initiation of gastric cancer. *World J Gastroenterol* 2017; 23(33): 6119-6127 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6119.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6119>

INTRODUCTION

Gastric cancer (GC) accounts for a large quantity of cancer-related deaths^[1]. Over the decades, the prognosis of GC patients has improved, while overall survival rate of patients with GC has not been improved significantly yet, especially for advanced GC patients^[2-4], which might be attributed to the poor understanding of mechanisms underlying the initiation and progression of GC. It is widely accepted that GC is a complex, heterogeneous, and multistep disease caused by various factors encompassing *Helicobacter pylori* infection, heredity, living habits, etc., which trigger series of molecular alterations, such as inactivation of tumor suppressor genes, activation

of oncogenes, telomerase activation, DNA damage and even genome instability^[5-8]. DNA-PKcs is the catalytic subunit of the DNA-dependent protein kinase and can combine with the Ku 70/Ku 80 heterodimer (Ku 70/80) into the DNA-dependent protein kinase (DNA-PK), which plays a crucial role in the activation of the non-homologous end joining (NHEJ) pathway^[9,10]. Moreover, ERGIC1 has been identified as a cycling protein and participates in membrane trafficking and selective transport of cargo between the endoplasmic reticulum (ER), the intermediate compartment (ERGIC), and the Golgi apparatus in HepG2 cells, suggesting a possible role in protein secretion out of the ER^[11].

Currently, high-throughput sequencing technologies, such as microarray assay and next-generation sequencing, have helped researchers obtain much more insight into molecular alterations^[12]. These new tools have given a huge impetus to the discovery of novel intracellular molecular pathways and molecular subtypes of GC. For instance, some researchers have recommended that GC should be classified into Epstein-Barr virus-positive subtype, microsatellite instability subtype, genomically stable subtype, and chromosomally unstable subtype on the basis of the spectra of genetic alterations related with relevant clinical features, which is a totally new classification method^[7,12].

However, it has been reported that GC is characterized by subtype heterogeneity and a lack of consistent genomic alterations across different individuals^[13]. Furthermore, due to different translation regulation, posttranslational modifications and stability of proteins, genetic conditions cannot always provide reliable protein expression patterns, suggesting that it may be not sufficient to take advantage of the gene sequence-targeted tools to explore the molecular mechanisms underlying the GC initiation and progression^[14]. Thus, proteomics has been thrust into the spotlight for its accurate and direct presentation of protein expression patterns that can provide much more information about the cellular function or dysfunction compared with genetic analysis^[15]. It has been widely accepted that GC initiation can be divided into five stages including normal gastric mucosa, atrophic gastritis with mild dysplasia, atrophic gastritis with moderate dysplasia, atrophic gastritis with severe dysplasia, and mucosal GC. To date, abnormal expression of many proteins has been reported to be involved in GC carcinogenesis in several GC proteomic studies^[12]. However, all of the previous studies only focused on identifying the differences between normal gastric epithelial tissues and GC rather than investigating the differences of protein among mild dysplasia, moderate dysplasia, severe dysplasia, and mucosal GC, which may contribute to more accurate insight into the molecular mechanisms underlying GC initiation. Thus, in this study we collected specimens of normal gastric mucosa, gastric mucosa with mild

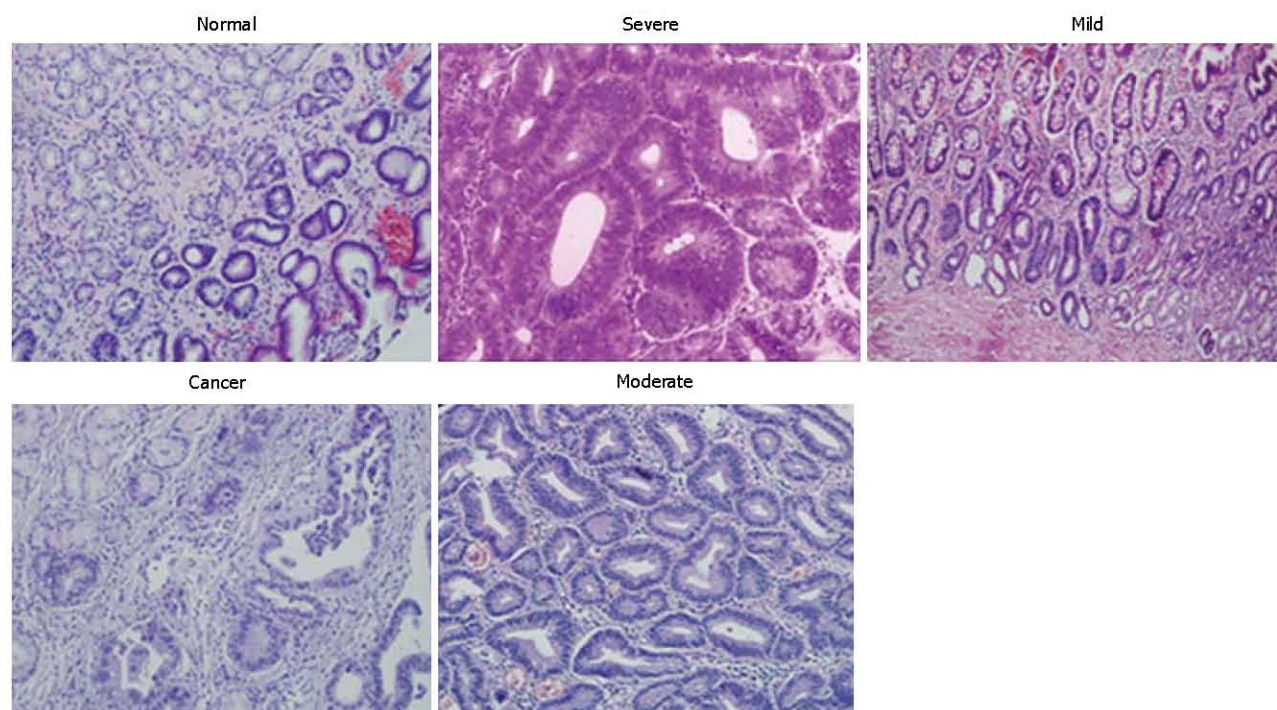


Figure 1 Representative graphs of HE staining (20 \times) of various gastric tissues including dysplasia.

Table 1 Basic demographic data of the study subjects

Group	n	Age (range) (yr)	Male/female
Healthy	30	43 (38-51)	16/14
Mild dysplasia	30	54 (42-67)	22/18
Moderate dysplasia	30	58 (40-65)	14/16
Severe dysplasia	30	63 (43-76)	19/11
Gastric cancer	40	61 (39-68)	23/17

dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC, and utilized label-free quantification technology integrated with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify differentially expressed proteins in the five kinds of specimens in order to determine the molecular mechanisms underlying GC initiation, which may promote potential preventive and therapeutic strategies for GC. We also focused on ERGIC1 and DNA-PKcs proteins for their inverse expression patterns identified in the proteomic analysis.

MATERIALS AND METHODS

Clinical specimens

Clinical specimens were collected at the Second Hospital of Lanzhou University from 2010 to 2015. A total of 160 patients were enrolled in this study, including 30 patients with a normal gastric mucosa, 30 patients with atrophic gastritis accompanied by mild dysplasia, 30 patients with atrophic gastritis accompanied by moderate dysplasia, 30 patients with atrophic gastritis accompanied by severe dysplasia, and 40 patients

with early intestinal-type GC (Table 1 and Figure 1). All patients underwent upper gastroduodenoscopy or gastrectomy and were pathologically reviewed and examined. This study was approved ethically by the Second Hospital of Lanzhou University. All of the patients provided written informed consent to participate in the study.

Proteomic analysis of gastric samples

Sample preparation: The five kinds of formalin-fixed, paraffin-embedded (FFPE) specimens were divided into three groups, respectively. Each FFPE specimen was cut into six pieces at a thickness of at least 4 μ m, in order to ensure that the protein quantity to detect was no less than 40 μ g. Fifteen groups of FFPE specimens were deposited in liquid nitrogen after conventional dewaxing, gradient alcohol, and hydration for proteomic analysis.

After 2 μ L of SDT buffer was added, the sample was ground with a pestle and a mortar. The homogenate was sonicated and then boiled for 15 min. After centrifugation at 14000 g for 40 min, the supernatant was filtered with 0.22 μ m filters. The protein content of the filtrate was quantified with the BCA Protein Assay Kit (Bio-Rad, the United States), and the sample was then stored at -80 $^{\circ}$ C.

SDS-PAGE separation: Samples (40 μ g proteins) were mixed with 5 \times loading buffer and boiled for 5 min. The proteins were separated on a 12.5% SDS-PAGE gel at a constant current of 14 mA for 90 min. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining.

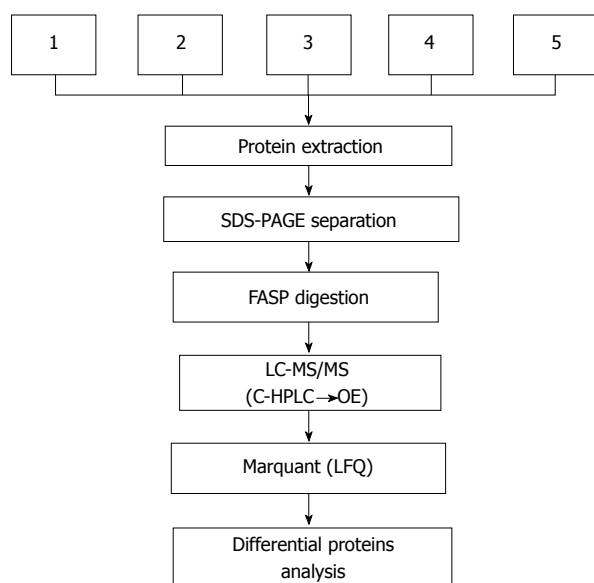


Figure 2 Outline of proteomic experimental workflow. SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; C-HPLC: Capillary-high performance liquid chromatography; QE: Q-exactive; LFQ: Label free quantitative.

Filter-aided sample preparation (FASP digestion):

A total of 100 μg proteins for each sample were added into 30 μL SDT buffer (4% SDS, 100 mmol/L DTT, 150 mmol/L Tris-HCl, pH 8.0). The detergent, DTT and other low-molecular-weight components were removed using UA buffer (8 mol/L urea, 150 mmol/L Tris-HCl pH 8.0) by repeated ultrafiltration (Microcon units, 10 kDa). Then, 100 μL of iodoacetamide (100 mmol/L IAA in UA buffer) was added to block reduced cysteine residues and the samples were incubated for 30 min in darkness. The filters were washed with 100 μL of UA buffer three times and then 100 μL of 25 mmol/L NH_4HCO_3 buffer twice. Finally, the protein suspensions were digested with 4 μg of trypsin (Promega) in 40 μL of 25 mmol/L NH_4HCO_3 buffer overnight at 37 $^\circ\text{C}$, and the resulting peptides were collected as a filtrate. The peptides of each sample were desalted on C18 Cartridges (Empore SPE Cartridges C18 (standard density), bed I.D. 7 mm, volume 3 mL, Sigma), concentrated by vacuum centrifugation, and reconstituted in 40 μL of 0.1% (v/v) formic acid. The peptide content was estimated by UV light spectral density at 280 nm using an extinction coefficient of 1.1 of 0.1% (g/L) solution that was calculated on the basis of the frequency of tryptophan and tyrosine in vertebrate proteins.

Mass spectrometry: Each fraction was injected for nanoLC-MS/MS analysis. The peptide mixture was loaded onto a reverse phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm \times 2 cm, nanoViper C18) connected to a C18-reversed phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in

buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nL/min controlled by IntelliFlow technology (one hour gradient: 0%-35% buffer B for 50 min, 35%-100% buffer B for 5 min, and held in 100% buffer B for 5 min).

LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) that was coupled to Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific) for 120 min. The mass spectrometer was operated in positive ion mode. MS data were acquired using a data-dependent top10 method that dynamically chooses the most abundant precursor ions from the survey scan (300-1800 m/z) for high energy collision dissociation (HCD) fragmentation. Automatic gain control target was set at 3×10^6 , and maximum inject time set at 10 ms. Dynamic exclusion duration was 40.0 s. Survey scans were acquired at a resolution of 70000 at m/z 200. The resolution for HCD spectra was set at 17500 at m/z 200, and isolation width was 2 m/z. Normalized collision energy was 30 eV and the underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was run with peptide recognition mode enabled (Figure 2).

Data analysis: The MS data were analyzed using MaxQuant software version 1.3.0.5 (Max Planck Institute of Biochemistry in Martinsried, Germany).

Immunohistochemistry

Primary antibodies were used in this study as follows: mouse monoclonal anti-human DNA-PKcs (GR190339-3; Abcam, Cambridge, United Kingdom) and anti-human ERGIC1 (GR104221-6; Anbobia, San Francisco, CA, the United States). The anti-human DNA-PKcs antibody was diluted 1:400, and the anti-human ERGIC1 antibody diluted 1:3500. The paraffin sections were mounted on slides, dewaxed in xylene, and sequentially dehydrated in 100%, 95% and 85% ethanol. The sections were stained using the PV-6000 Polymer Detection System (Zhongshan Goldenbridge, Beijing, China). Initially, endogenous peroxidase was blocked using 3% H_2O_2 . After the sections were incubated with the primary antibody overnight at 4 $^\circ\text{C}$, they were washed with PBS and then incubated with polymer helper for 30 min and poly peroxidase-anti-mouse/rabbit IgG for 30 min. After that, the sections were washed with PBS, and then incubated with 3,3'-diaminobenzidine (DAB, Zhongshan Goldenbridge). The sections incubated with PBS without primary antibodies were used as negative controls. Finally, the sections were counterstained with hematoxylin and examined under a light microscope. The cells that were stained a yellow or brown color in the nucleus and/or cytoplasm were defined as positive. Five randomly selected fields per section were analyzed. In a ran-

Table 2 Results of DNA-PKcs and ERGIC1 from proteomic analysis

Protein ID	Gene	Protein	Fold change			
			Mild/normal	Moderate/normal	Severe/normal	Cancer/normal
P78527	PRKDC	DNA-dependent protein kinase catalytic subunit	1.12	1.54	1.60	2.30
Q969X5	ERGIC1	Endoplasmic reticulum-Golgi intermediate compartment protein 1	1.18	0.84	0.73	0.46

Table 3 Results of DNA-PKcs and ERGIC1 from immunohistochemistry

Group	n	DNA-PKcs				n	ERGIC1			
		-	+	++	+++		-	+	++	+++
Normal	30	12	18	0	0	30	2	2	2	24
Mild	30	14	16	0	0	30	2	3	3	22
Moderate	30	5	3	23	0	30	2	3	25	0
Severe	30	3	2	25	0	30	5	5	20	0
Cancer	30	0	2	6	22	40	9	27	4	0

domly selected field from representative areas, the immunoreactive cells among 100 cells were assessed and quantified by percentage. Then, the average percentage of the five fields was used to assess the area of immunostaining (0 = 0%-5%; 1 = 6%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 = 76%-100%). In addition, the intensity of immunostaining was also semi-quantitatively assessed (0 = negative, 1 = weak, 2 = moderate, 3 = intense). Then, the scores from the "area × intensity" were calculated and used to describe the overall staining intensity that semi-quantitatively reflects the overall expression levels of proteins. The overall staining intensity was scored as follows: negative (-): 0-2; mild (+): 3-5; moderate (++) : 6-8; and strong (+++) : 9-12. All the sections were assessed and scored by two pathologists who were blinded to the clinical data of the patients.

Statistical analysis

All the data are expressed as mean ± SD or percentage. The χ^2 test (SPSS v.16.0 for Windows; SPSS, Inc, Chicago, IL, United States) was used to evaluate the difference between categorical variables. A *P*-value less than 0.05 was considered statistically significant.

RESULTS

Differentially expressed proteins detected by LC-MS/MS

To identify differentially expressed proteins in normal gastric mucosa, atrophic gastritis with mild dysplasia, atrophic gastritis with moderate dysplasia, atrophic gastritis with severe dysplasia, and early mucosal GC, we performed a study with label-free quantification technology integrated with LC-MS/MS. A total of 17443 peptides matching 2807 proteins were identified from tissue analysis. The expression of a total of 365 proteins, with a threshold of a 1.2-fold change and a *P*-value less than 0.05 between mild dysplasia, moderate dysplasia, severe dysplasia or early mucosal GC and matched normal gastric mucosa tissues, were considered to be statistically significantly different.

Aberrant expression of DNA-PKcs and ERGIC1 revealed by proteomic analysis

The fold changes in mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC compared to matched normal gastric mucosa tissues were 1.12, 1.54, 1.6, and 2.3 for DNA-PKcs, and 1.18, 0.84, 0.73, and 0.46 for ERGIC1, respectively (Table 2).

Aberrant expression of DNA-PKcs and ERGIC1 revealed by immunohistochemistry

In order to confirm the results of DNA-PKcs and ERGIC1 from proteomic analysis, we further examined the expression of DNA-PKcs and ERGIC1 by immunohistochemistry. The strongly positive (+++) rates in normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC were 80% (24/30), 73% (22/30), 0% (0/30), 0% (0/30), and 0% (0/40), respectively, for ERGIC1, and for DNA-PKcs the strongly positive (+++) staining was observed only in the sections of early mucosal GC. The moderately positive (++) rates in normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC were 6.6% (2/30), 6.6% (3/30), 83% (25/30), 67% (20/30), and 10% (4/40), respectively, for ERGIC1, and 0% (0/30), 0% (0/30), 76% (23/30), 83% (25/30), and 20% (6/30), respectively, for DNA-PKcs (Figures 3 and 4). The details on the rates of different-grade overall staining intensity are presented in Table 3.

In general, the average immunohistochemistry scores of the DNA-PKcs protein significantly increased, while those of ERGIC1 protein significantly decreased along the sequence of normal gastric mucosa, mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC.

DISCUSSION

Using label-free quantification technology combined with LC-MS/MS, the expression of 365 proteins based on the tendency of fold change was revealed to be

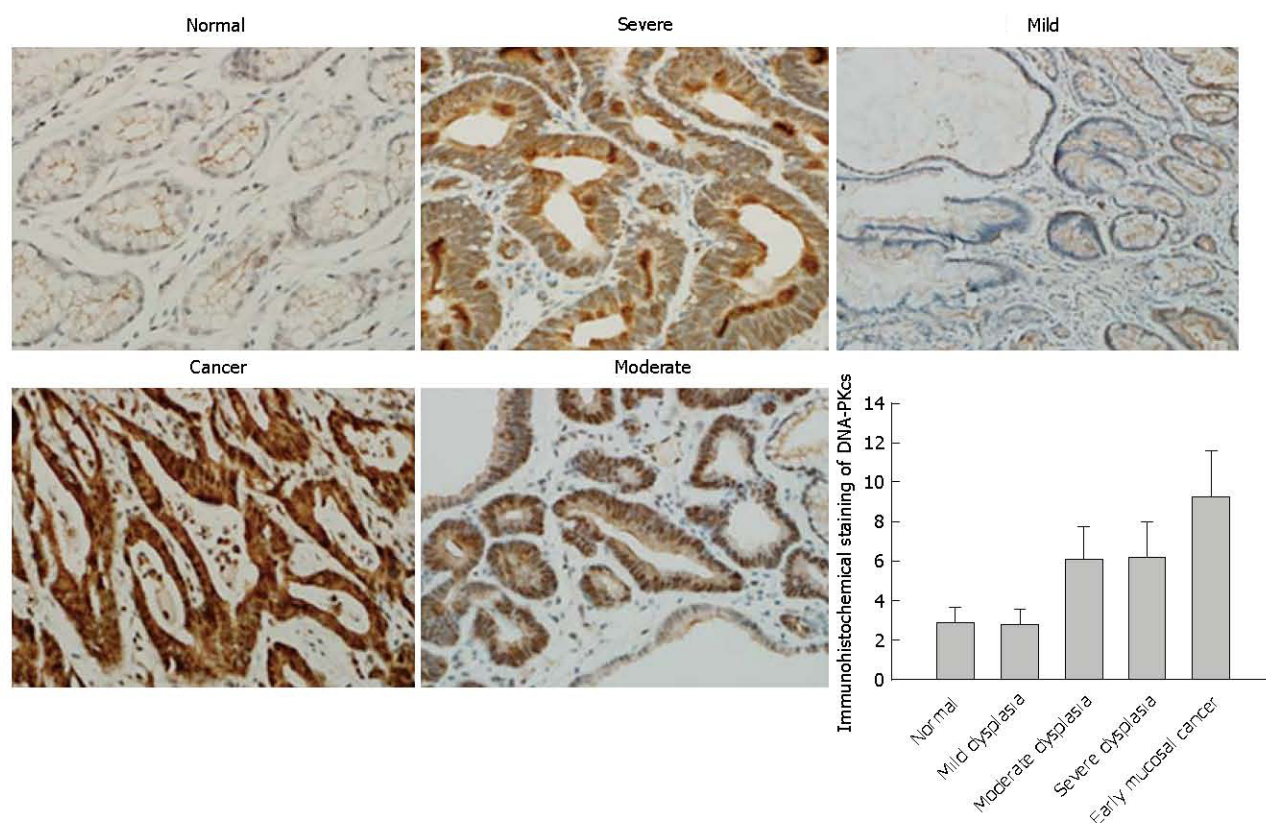


Figure 3 Immunohistochemical staining of DNA-PKcs (40 ×). A: Normal; B: Mild dysplasia; C: Moderate dysplasia; D: Severe dysplasia; E: Early mucosal cancer.

statistically different between various stages of GC initiation. Furthermore, we observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change, suggesting that abnormal ERGIC1 and DNA-PKcs expression may play an important role in GC initiation.

It has been reported that DNA-PKcs plays an important role in the activation of the NHEJ pathway^[9,10]. When a DNA damage signal in cells is sent out, DNA-PKcs will recognize it and initiate a damage response in the first place, followed by the event that Ku 70/80 binds to the damaged DNA ends and induces DNA-PKcs to form DNA-PK that triggers NHEJ repair activities^[16]. NHEJ is an error-prone and non-specific DNA repair mechanism and can be induced before homologous recombination, whose excessive activation has the capability of regulating cell cycle arrest, cell apoptosis, chromosome recombination, and genome instability, all of which are closely related with carcinogenesis^[17,18]. Dysregulation of DNA-PKcs has been reported to be associated with pathological processes in various cancers^[19]. Consistent with the previous study indicating that DNA-PKcs expression in GC was up-regulated compared with normal gastric mucosa, and associated with GC progression^[20], up-regulation of DNA-PKcs expression was also found in our study. Furthermore, our study showed more information that DNA-PKcs

expression increased along with different stages of GC initiation. Hence, it is possible to hypothesize that enhanced NHEJ resulting from the overexpression of DNA-PKcs may contribute to GC initiation and progression.

ERGIC1, a cycling protein, plays a part in protein secretion out of the ER by participating in membrane trafficking and selective transport of cargo between the ER, the intermediate compartment, and the Golgi apparatus^[11]. ER stress is defined as a disequilibrium between protein folding ability of the ER and protein load, causing the accumulation of misfolded and unfolded proteins^[21]. Many factors, including hypoxia, starvation, infections, changes in secretory needs and so on, throw a challenge to the folding capacity of the cell and subsequently trigger ER stress^[22]. ER stress has been speculated to be involved in many cancers including GC^[23]. ER stress is capable of activating NF- κ B, ROS, and JNK signal pathways^[24-27], resulting in chronic inflammatory response that contributes to carcinogenesis. Besides, accumulating evidence indicated that ER stress response could facilitate cancer progression probably by promoting tumor angiogenesis and malignant cell autophagy, and mitigating apoptosis^[28-31]. In this study, we observed that ERGIC1 expression decreased gradually along with different stages of GC initiation. Down-regulation of ERGIC1 expression may affect the protein secretion function of the ER and then disturb ER homeostasis,

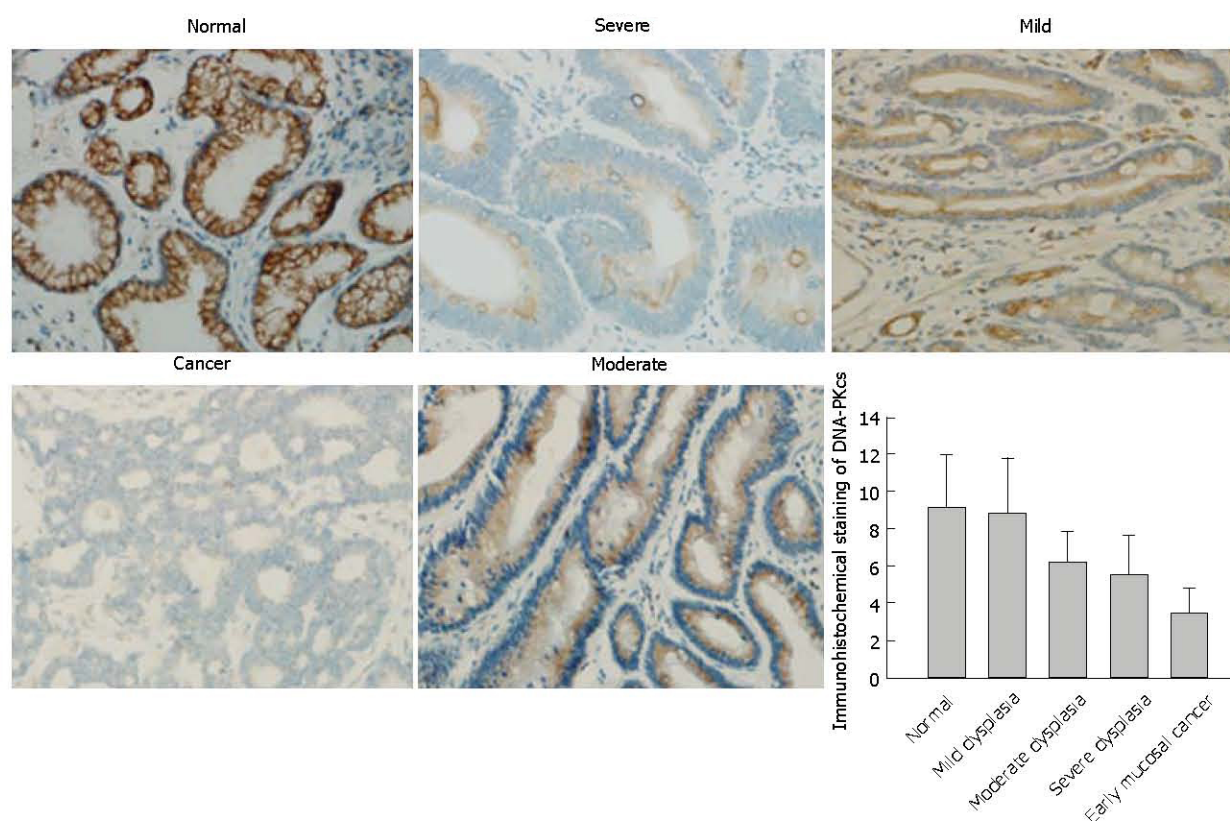


Figure 4 Immunohistochemical staining of ERGIC1 (40 ×). A: Normal; B: Mild dysplasia; C: Moderate dysplasia; D: Severe dysplasia; E: Early mucosal cancer.

which may cause the accumulation of unfolded and misfolded proteins, finally resulting in ERS. Thus, it is possibly reasonable to speculate that down-regulation of ERGIC1 expression may contribute to GC initiation by promoting ERS in gastric mucosal epithelial cells. Besides, some studies suggested that ER stress response can also induce the apoptosis of malignant cells^[32-34], indicating that ER stress response may play a dual role in cancers. Furthermore, a study by Vainio *et al.*^[35] indicated that ERGIC1 expression was up-regulated in prostate cancer samples and knockdown of ERGIC1 could inhibit the ERG oncogene expression in prostate cancer cells *in vitro*, which suggested that the change tendency of ERGIC1 expression in cancers may be different, depending on the genomic or biological function of cancer cells. Therefore, another possible molecular mechanism responsible for the role of down-regulation of ERGIC1 in the GC initiation is that down-regulation of ERGIC1 perhaps disturbs the expression of some oncogenes or anti-oncogenes. Additionally, despite that there are some specific relationships between ERGIC1 and DNA-PKcs based on the inverse expression patterns observed in the current study, we did not provide adequate evidence to confirm that. Thus, further studies are required to elucidate the mechanisms by which down-regulation of ERGIC1 contributes to the initiation and progression of GCs and the internal link between ERGIC1 and DNA-PKcs.

In conclusion, our study conducted a systematic proteomic analysis of normal gastric mucosa, gastric mucosa with mild dysplasia, gastric mucosa with moderate dysplasia, gastric mucosa with severe dysplasia and early mucosal GC, which covered the whole sequential process of GC initiation, and suggested that the aberrant DNA-PKcs and ERGIC1 expression may be involved in the initiation of GC.

COMMENTS

Background

Gastric cancer (GC) is a global malignant disease with high incidence and mortality. The carcinogenesis of GC has been defined as a biological process involving polygene-driven, multi-step, and multi-stage events. However, the molecular mechanisms underlying the carcinogenesis of GC remain unknown.

Research frontiers

Using proteomics analysis, the current study aimed to investigate the molecular mechanisms of GC carcinogenesis.

Innovations and breakthroughs

The authors first report that aberrant ERGIC1 and DNA-PKcs expression may be involved in GC initiation.

Applications

Using label-free quantification technology combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS), the authors observed that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation based on the tendency of fold change. The expression patterns of ERGIC1 and DNA-PKcs revealed by

immunohistochemistry were consistent with the LC-MS/MS results.

Peer-review

The authors demonstrated that that ERGIC1 expression decreased, while DNA-PKcs expression increased gradually along with different stages of GC initiation. A total of 160 specimens were enrolled in this study to identify differentially expressed proteins in normal gastric mucosa, gastric mucosa with mild dysplasia, moderate dysplasia, severe dysplasia, and early mucosal GC. These results are interesting and very valuable to verify the molecular mechanisms of GC carcinogenesis.

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Retrospective Cohort Study

Real-world treatment patterns of gastrointestinal neuroendocrine tumors: A claims database analysis

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Institutional review board statement: We conducted a retrospective cohort study using the Truven Health Analytics MarketScan Database and the IMS Health Pharmetrics Database, both commercial health insurance claims database for employer-insured beneficiaries in the United States. The databases are fully compliant with the Health Insurance Portability and Accountability Act and meets the criteria for a limited-use dataset. Since the patient and provider data included in this analysis were fully de-identified, this study was exempt from the Institutional Review Board review.

Conflict-of-interest statement: Cai B and Neary MP are employees of Novartis Pharmaceuticals Corporation; Benson III AB was paid as a research consultant for the study as a subject matter expert by Novartis and is an employee of Northwestern University; Broder MS, Chang E and Papoyan E are employees of Partnership for Health Analytic Research, LLC (PHAR, LLC), a health services research company paid by Novartis to conduct this research.

Data sharing statement: The study statistician, Eunice Chang, conducted all statistical analysis for this study using Health Insurance Portability and Accountability Act-compliant commercial-insurance secondary databases MarketScan and PharMetrics.

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Abstract

AIM

To describe real-world treatment patterns of gastrointestinal neuroendocrine tumors (GI NET).

METHODS

In this retrospective cohort study, we used 2009-2014 data from 2 United States commercial claims databases to examine newly pharmacologically treated patients using tabular and graphical techniques. Treatments included somatostatin analogues (SSA), cytotoxic chemotherapy (CC), targeted therapy (TT), interferon

(IF) and combinations. We identified patients at least 18 years of age, with ≥ 1 inpatient or ≥ 2 outpatient claims for GI NET who initiated pharmacologic treatment from 7/1/09-6/30/14. A 6 mo clean period prior to first treatment ensured patients were newly treated. Patients were followed until end of enrollment or the study end date, whichever was first.

RESULTS

We identified 2258 newly treated GI NET patients: mean (SD) age was 55.6 years (SD = 9.7), 47.2% of the patients were between 55 and 64 years, and 48.8% were female. All regions of the United States were represented. 59.6% started first-line therapy with SSA monotherapy (964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide), 33.3% CC, 3.6% TT, and 0.5% IF. The remainder received combinations. Mean follow up was 576 d. Overall mean first-line therapy duration was 361 d (449 d for SSA, 215 for CC, 267 for TT). 58.9% of patients had no pharmacological treatment beyond first line. The most common second-line was combination therapy with SSA. In graphical pattern analysis, there was no clear pattern visible after first line therapy.

CONCLUSION

In this study, 60% of patients initiated treatment with SSA alone or in combination. The relatively long time to discontinuation suggests possible sustained effectiveness and tolerability.

Key words: Gastrointestinal neuroendocrine tumors; Treatment patterns; Insurance claims; Somatostatin analogue; Targeted therapy; Chemotherapy

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Core tip: In this retrospective study of real-world treatment patterns, somatostatin analogues were the most common initial pharmacologic treatment in patients with gastrointestinal neuroendocrine tumors, and most of the remaining patients began treatment with chemotherapy. However, despite the many treatment options, over half of the patients discontinued treatments after first line and only less than 10% of patients received any second-line pharmacotherapy. Given limitations of claims data to elucidate reasons for this lack of continued treatment, a study using more detailed clinical information such as medical charts or physician surveys is warranted.

Benson III AB, Broder MS, Cai B, Chang E, Neary MP, Papoyan E. Real-world treatment patterns of gastrointestinal neuroendocrine tumors: A claims database analysis. *World J Gastroenterol* 2017; 23(33): 6128-6136 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6128.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6128>

INTRODUCTION

Neuroendocrine tumors (NET) comprise a broad family of rare and often slow growing malignancies. NET can develop anywhere in the body and arise from neuroendocrine cells throughout the endocrine system^[1,2]. Approximately two-thirds of NET tumors occur in the gastrointestinal (GI) tract. These sites include the stomach, small intestine, appendix, colon, and rectum^[3]. NET secrete peptides and neuroamines that cause distinct syndromes (e.g., carcinoid syndrome), in which case they are referred to as "functional" tumors. Clinical presentation depends on the site of the primary tumor and whether they are functional. Surgery may be curative in the early stages, but delayed diagnosis is typical.

While rare, the incidence and prevalence of NET appear to be increasing worldwide^[4-8]. The incidence of NET in the United States increased from 10.9 cases per million person-years (PMPY) in 1973 to 52.5 PMPY in 2004, and to 69.8 PMPY in 2012 as reported using the United States Surveillance Epidemiology and End Results database^[4,9]. Prevalence also increased and was reported as 216 per million per year for GI NET in the United States.

The management of GI NET is based on a variety of factors including stage, anatomic location, and the presence and type of symptoms. The most recent NCCN guidelines for unresectable and metastatic GI NET recommend somatostatin analogues (SSA) as first-line treatment, but do not recommend a particular treatment sequence for the remaining therapies^[10]. Considering the heterogeneity of GI NET tumors and the resultant lack of specificity in guidelines, we aimed to describe the current real-world treatment patterns of GI NET in a large sample of patients.

MATERIALS AND METHODS

Data source

We conducted a longitudinal, retrospective cohort analysis of newly pharmacologically treated GI NET patients using two large United States commercial claims databases. Data from the Truven Health Analytics MarketScan database and the IMS Pharmetrics database (both using dates from January 1, 2009 to December 31, 2014) were combined to increase sample size. To prevent duplicate records, patients with the same age, gender, region, and date of first GI NET diagnosis in a calendar year found in both databases were randomly removed from one of the databases. Both databases are Health Insurance Portability and Accountability Act compliant administrative claims databases that contain de-identified adjudicated medical claims (e.g., inpatient and outpatient services) and pharmacy claims (e.g., outpatient prescriptions) submitted for payment by

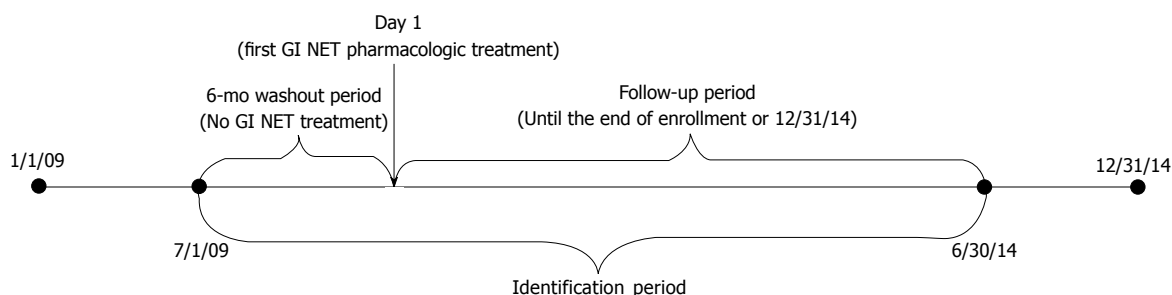


Figure 1 Study timeline. The first gastrointestinal neuroendocrine tumors (GI NET) pharmacologic treatment claim on or after the appearance of the GI NET diagnosis code and within the ID period (7/1/2009 to 6/30/2014) was considered to be the index date. Patients were required to be enrolled for a baseline period of at least six months before the index date. Patient follow-up was variable and continued until the end of enrollment or the study end date (12/31/14), whichever was first.

providers, healthcare facilities, and pharmacies. For both data sources, claims include information on each physician visit, medical procedure, hospitalization, drug dispensed, date of service, number of days of medication supplied, test performed, and complete payment information. Each medical claim has a principal diagnosis and secondary diagnoses codes associated with it. Available patient demographic information includes age, gender, and geographic region. Dates of enrollment and disenrollment are also recorded. As the data were fully de-identified, this study was considered exempt from approval by the Institutional Review Board.

Cohort selection

Patients at least 18 years of age were identified from each dataset if they had at least 1 inpatient or 2 outpatient claims with an International Statistical Classification of Disease-9-Clinical Modification (ICD-9-CM) for GI NET (209.00-209.03, 209.10-209.17, 209.23, 209.24-209.27, 209.40-209.43, 209.50-209.57, 209.62, 209.65-209.67) during the study period (1/1/2009-12/31/2014). The first GI NET pharmacologic treatment claim on or after the appearance of the GI NET diagnosis code and within the ID period (7/1/2009 to 6/30/2014) was considered to be the index date. Patients were required to be enrolled for a baseline period of at least six months before the index date. To ensure new treatment, patients with any evidence of pharmacologic treatment during this baseline period were excluded. In order not to include the same patient twice, we searched for any patients with the same age, gender, region, and date of GI NET diagnosis who could be found in both databases, but we found none. Patient follow-up was variable and continued until the end of enrollment or the study end date (12/31/2014), whichever was first (Figure 1).

Study variables and measures

The primary outcome measure was the use of pharmacologic or liver directed therapy. Pharmacotherapy was divided into four groups: SSA, TT, CC and IF. SSA included octreotide and lanreotide, TT included everolimus and sunitinib, and CC included

temozolomide, streptozotocin, doxorubicin, liposomal doxorubicin, fluorouracil, capecitabine, dacarbazine, oxaliplatin and thalidomide. Pharmacologic therapy was identified in claims using both the Healthcare Common Procedure Coding System (HCPCS) and National Drug Codes (NDC). Liver directed therapies comprised liver resection, transplant, lesion ablation (using radiotherapy, cryotherapy, microwave and thermal energy, and including laparoscopic, open and percutaneous routes), embolization (including bland, radioisotope, and chemotherapy), and radiation therapy. Liver directed therapies were identified in claims using HCPCS, ICD-9-CM, and Current Procedural Terminology (CPT) codes. Chemotherapy observed only once and on the same date as embolization was considered chemoembolization and not part of a pharmacologic regimen.

First-line therapy was defined as the pharmacologic treatment regimen observed on, or within three months of, the index date. Therapy included monotherapy or combination therapies. A three-month period after the index date was used to identify pharmacologic therapy intended as first-line but not administered on the index date. This would include, for example, combination chemotherapy where the second agent is given after some delay. Second-line therapy was defined as beginning when treatment was switched from one category of pharmacotherapy to another (*e.g.*, from SSA alone to CC alone), or when a new category of treatment was added (*e.g.*, from SSA alone to SSA plus CC). Changes from one cytotoxic agent to another, or one SSA to another, were not considered a switch. The first day of treatment switch or addition was defined as the initiation date of second-line therapy.

Statistical analysis

Means and proportions were presented in tabular analyses. An inverse Kaplan-Meier curve was used to show duration of first-line therapy. All data transformations and statistical analyses were performed using SAS[®] version 9.4 (SAS Institute, Cary, NC). Graphical analyses were conducted using GRAPHx[™], a proprietary graphics-based algorithm. The GRAPHx method uses multi-colored line segments to represent

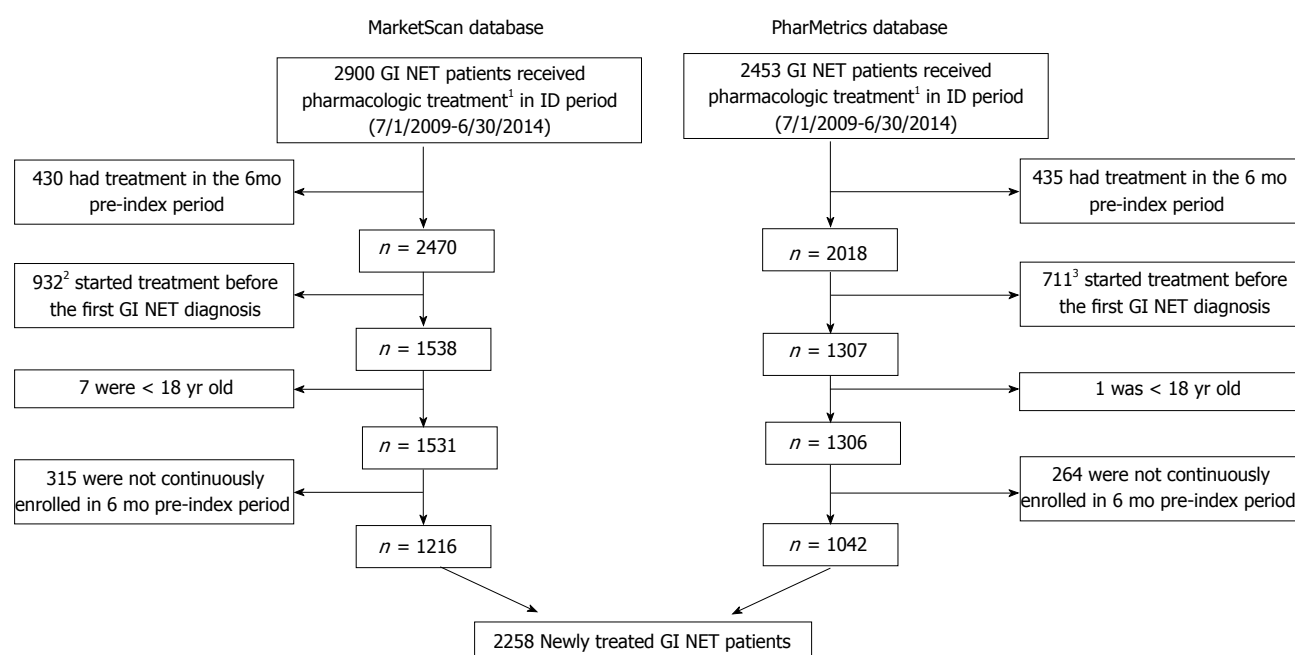


Figure 2 Patient identification. There were 2900 and 2453 gastrointestinal neuroendocrine tumors (GI NET) patients who also had a claim for pharmacologic treatment between 7/1/2009 and 6/30/2014 in the MarketScan and PharMetrics databases, respectively. After excluding patients who had treatment during a 6-mo pre-index period (and therefore were considered to be continuing, rather than initiating, treatment); received treatment before receiving a diagnosis of GI NET; were < 18 yr old; or were not continuously enrolled in the 6-mo pre-index period, there remained 2258 newly treated GI NET patients who were included in the study. ¹Somatostatin analogues (SSAs), targeted therapy, cytotoxic chemotherapy, or interferon; ²324 (34.8%) within 3 mo, and 516 (55.4%) within 6 mo; ³249 (35.0%) within 3 mo, and 380 (53.4%) within 6 mo.

various treatments, plotting them over time. The images are reviewed visually for the presence and length of segments and change in colors and patterns over time.

RESULTS

There were 2900 and 2453 patients meeting the definition of GI NET who also had a claim for pharmacologic treatment between 7/1/2009 and 6/30/2014 in the MarketScan and PharMetrics databases, respectively. After excluding patients who had treatment during a 6-mo pre-index period (and therefore were considered to be continuing, rather than initiating, treatment); received treatment before receiving a diagnosis of GI NET; were < 18 years old; or were not continuously enrolled in the 6-mo pre-index period, there remained 2258 newly treated GI NET patients who were included in the study (Figure 2).

Gender was evenly split with $n = 1103$ (48.8%) female patients and $n = 1155$ (51.2%) male. The average age was 55.6 years (SD = 9.7) and 47.2% of the patients were between 55 and 64 years. All regions of the United States were represented. More than half of patients, $n = 1345$ (59.6%), were treated with SSA as first-line monotherapy, 964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide. An additional 75 patients (3.3%) received SSA in combination with other either CC, TT or IF. The second largest group, $n = 752$ (33.3%), was treated with CC monotherapy, and $n = 81$ (3.6%) received TT monotherapy (Table 1).

Mean duration of first-line therapy was 361 d (SD = 385) for all newly treated patients. The mean observed duration of treatment for first-line SSA monotherapy users was 449 d (SD = 434.2). It was 215 d (SD = 228.8) for first-line CC monotherapy and 267 d (SD = 325.7) for first-line TT monotherapy (Table 2). By 588 d of treatment (1.61 years), half of SSA initiators had discontinued treatment, compared to 182 d (0.498 years) for half of CC users and 171 d (0.47 years) for half of TT users to discontinue treatment (Figure 3). Liver directed therapy was used by 12.5% during first-line pharmacologic therapy; another 3.7% received it sometime after the first-line (Table 2).

By the end of the study follow-up period [mean (SD, median) of 576 d (447.1, 454)] 58.9% ($n = 1331$) patients had stopped pharmacologic therapy completely. These patients continued to be enrolled in one of the databases but no longer had claims for pharmacologic treatment. In Figure 4, these patients can be identified as colored line segments that terminate in gray segments of variable length, with the gray representing the period of no treatment. An additional 32.7% ($n = 738$) continued their initial therapy until the end of their enrollment; these patients were still receiving their first-line therapy at the time they left a covered plan or reached the end of study. This pattern is shown in Figure 4 as a colored segment terminating in white. The remaining 8.4% ($n = 189$) were observed to change pharmacologic treatment during the follow-up period (a colored segment terminating in different colored segment).

Table 1 Patient demographics by first-line treatment *n* (%)

	First-line treatment						All newly treated patients		
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	SSA + IF	SSA + TT + CC
<i>n</i>	1345 ¹	752	81	42	31	3	2	1	1
Age (mean ± SD, yr)	59.6%	33.3%	3.6%	1.9%	1.4%	0.1%	0.1%	0.0	0.0
18-24	56.3 ± 9.5	54.7 ± 9.9	54.9 ± 10.5	53.5 ± 10.9	53.8 ± 10.1	59.3 ± 3.8	58.5 ± 3.5	N/A	N/A
25-34	6 (0.4)	2 (0.3)	0 (0)	1 (2.4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
35-44	20 (1.5)	25 (3.3)	4 (4.9)	3 (7.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
45-54	121 (9.0)	92 (12.2)	9 (11.1)	4 (9.5)	4 (12.9)	0 (0)	0 (0)	0 (0)	0 (0)
55-64	371 (27.6)	225 (29.9)	18 (22.2)	12 (28.6)	12 (38.7)	0 (0)	0 (0)	0 (0)	0 (0)
65+	651 (48.4)	338 (44.9)	39 (48.1)	20 (47.6)	11 (35.5)	3 (100.0)	2 (100.0)	1 (100.0)	1 (100.0)
Female	176 (13.1)	70 (9.3)	11 (13.6)	2 (4.8)	4 (12.9)	0 (0)	0 (0)	0 (0)	0 (0)
Region	677 (50.3)	341 (45.3)	40 (49.4)	26 (61.9)	17 (54.8)	1 (33.3)	1 (50.0)	0 (0.0)	0 (0.0)
Midwest	321 (23.9)	183 (24.3)	20 (24.7)	13 (31.0)	7 (22.6)	0 (0)	1 (50.0)	0 (0)	0 (0)
Northeast	261 (19.4)	150 (19.9)	12 (14.8)	11 (26.2)	7 (22.6)	1 (33.3)	0 (0)	0 (0)	0 (0)
South	563 (41.9)	323 (43.0)	36 (44.4)	12 (28.6)	15 (48.4)	2 (66.7)	1 (50.0)	0 (0)	1 (100.0)
West	200 (14.9)	96 (12.8)	13 (16.0)	6 (14.3)	2 (6.5)	0 (0)	0 (0)	1 (100.0)	0 (0)
Year of treatment initiation	130 (9.7)	41 (5.5)	4 (4.9)	2 (4.8)	1 (3.2)	0 (0)	0 (0)	1 (100.0)	0 (0)
2009	271 (20.1)	122 (16.2)	4 (4.9)	11 (26.2)	7 (22.6)	0 (0)	1 (50.0)	0 (0)	0 (0)
2010	282 (21.0)	159 (21.1)	15 (18.5)	17 (40.5)	5 (16.1)	0 (0)	0 (0)	0 (0)	0 (0)
2011	270 (20.1)	168 (22.3)	33 (40.7)	4 (9.5)	10 (32.3)	0 (0)	1 (50.0)	0 (0)	1 (100.0)
2012	268 (19.9)	174 (23.1)	16 (19.8)	3 (7.1)	4 (12.9)	2 (66.7)	0 (0)	0 (0)	0 (0)
2013	124 (9.2)	88 (11.7)	9 (11.1)	5 (11.9)	4 (12.9)	1 (33.3)	0 (0)	0 (0)	0 (0)
Days of follow-up	621	514	454	588	425	244	675	836	496
Mean	(468.5)	(409.1)	(403.6)	(424.1)	(269.6)	(140.7)	(145.7)	N/A	N/A
SD	500	393	290	455	360	216	675	836	496
Median									

¹964 with octreotide LAR, 380 with octreotide SA, and 1 with lanreotide. SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

(Figure 4 and Table 2). Among these 189 patients who were observed to begin second-line therapy, 128 (67.7%) had initially been treated with SSA. Among these 128 first-line SSA users, 89 (69.5%) added additional therapy (e.g., CC or TT) as their second-line treatment. In patients who did not begin therapy with SSA, most received SSA monotherapy or combination therapy as second-line (Table 3). Liver directed therapy (short, red segments) appears dispersed throughout periods of both pharmacologic treatment (colored segments) and periods of no pharmacologic treatment (gray segments) (Figure 4). Among 1331 patients who stopped pharmacologic treatment and did not begin a second-line treatment, 5.5% were treated with liver-directed therapy within 30 d before or after they stopped.

DISCUSSION

This study used two very large, nationally representative claims databases, which together represent up to 100 million covered lives, to describe real-world treatment of GI NET. Three findings were of particular interest. First, the most common initial pharmacologic treatment was with SSA, with average duration of use of just over 18 mo. Second, although 60% of patients initiated treatment with SSA alone or in combination, most of the remainder began treatment with CC, therapy recommended

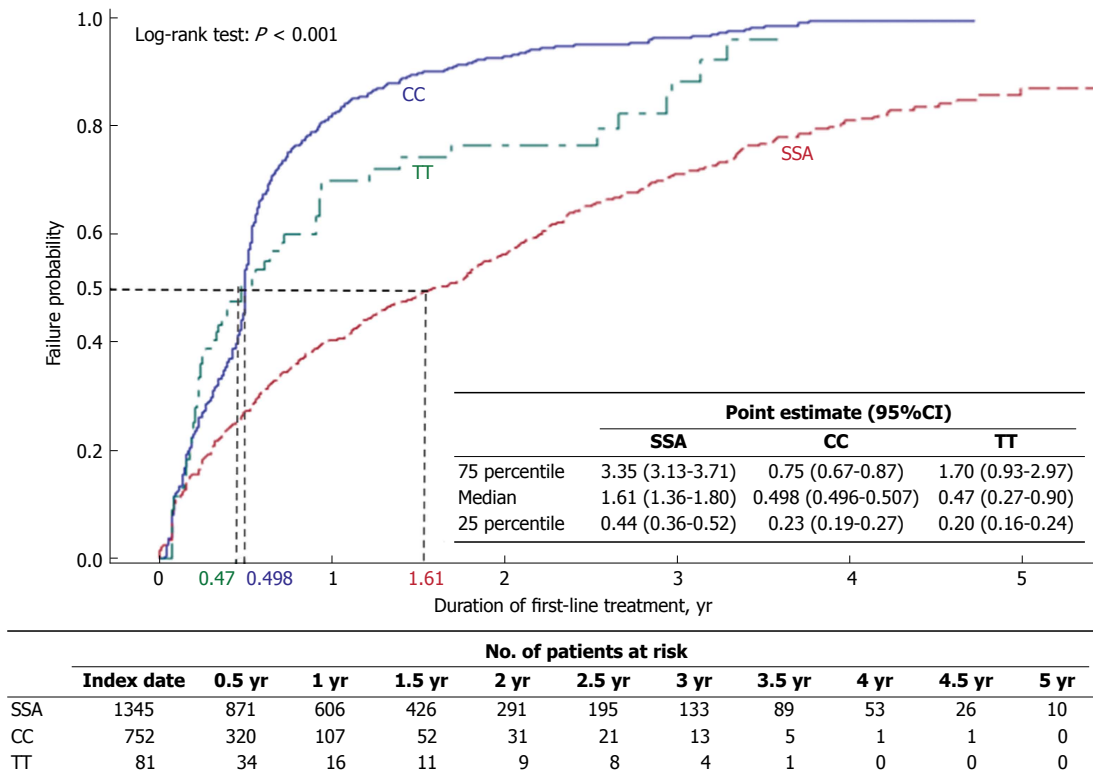
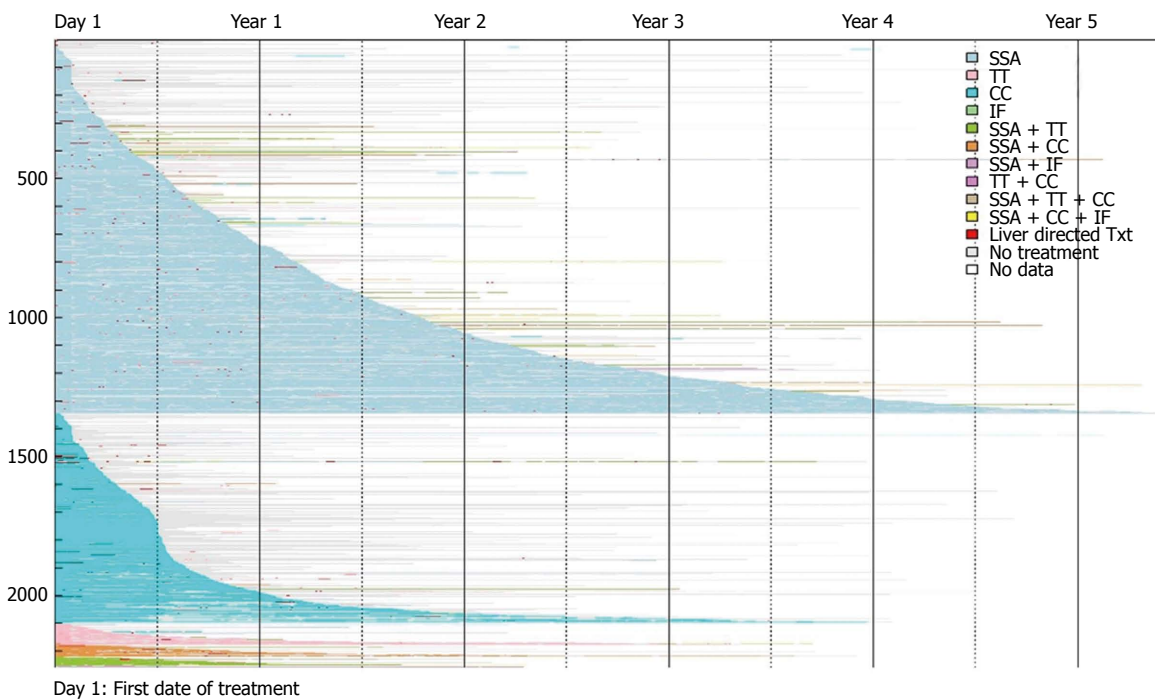


Figure 3 Time to discontinuation of first-line treatment. By 588 d of treatment (1.61 yr), half of SSA initiators had discontinued treatment, compared to 182 d (0.498 yr) for half of CC users and 171 d (0.47 yr) for half of TT users to discontinue treatment. SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy.



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Figure 4 Pharmacologic treatment. By end of study follow-up [mean (SD, median) of 576 d (447.1, 454)], 58.9% ($n = 1331$) patients had stopped pharmacologic therapy completely. These patients (no pharmacologic treatment claims, but remained enrolled in one of the databases) can be identified as colored line segments that terminate in gray segments of variable length, with the gray representing the period of no treatment. An additional 32.7% ($n = 738$) continued their initial therapy until the end of their enrollment; these patients were still receiving their first-line therapy at the time they left a covered plan or reached the end of study. This pattern is shown as a colored segment terminating in white. The remaining 8.4% ($n = 189$) were observed to change pharmacologic treatment during the follow-up period (a colored segment terminating in different colored segment). Liver directed therapy (short, red segments) appears dispersed throughout periods of both pharmacologic treatment (colored segments) and periods of no pharmacologic treatment (gray segments). SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

Table 2 Use of first-line treatment *n* (%)

	First-line						All newly treated patients		
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	SSA + IF	SSA + TT + CC
<i>n</i>	1345 59.6%	752 33.3%	81 3.6%	42 1.9%	31 1.4%	3 0.1%	2 0.1%	1 0.0%	1 0.0%
Duration of first-line treatment (mean ± SD, d)	449 ± 434.2	215 ± 228.8	267 ± 325.7	408 ± 327.9	276 ± 189.5	208 ± 165.6	251 ± 285.0	836 ± 0	426 ± 0
First-line ending status									
Stop	635 (47.2)	609 (81.0)	44 (54.3)	26 (61.9)	14 (45.2)	1 (33.3)	1 (50.0)	0 (0)	1 (100.0)
Switch	128 (9.5)	33 (4.4)	14 (17.3)	5 (11.9)	7 (22.6)	1 (33.3)	1 (50.0)	0 (0)	0 (0)
End of enrollment	582 (43.3)	110 (14.6)	23 (28.4)	11 (26.2)	10 (32.3)	1 (33.3)	0 (0)	1 (100.0)	0 (0)
Liver directed therapy timing									
During first-line	171 (12.7)	87 (11.6)	9 (11.1)	10 (23.8)	4 (12.9)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)
After first-line	36 (2.7)	39 (5.2)	7 (8.6)	0 (0.0)	2 (6.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
+/- 30 d after stopping first line therapy ¹	635 29 (4.6)	609 42 (6.9)	44 2 (4.5)	26 0 (0.0)	14 0 (0.0)	1 0 (0.0)	1 0 (0.0)	0 0 (0.0)	1 0 (0.0)

¹ Among patients who stopped.

by NCCN only if no other options (SSA, TT, or liver directed treatment) are feasible. Third, despite the many available treatment options, less than one in 10 patients was observed to receive treatment with second-line pharmacotherapy of any type.

Each of these findings must be considered in light of what is known about the disease as well as the inherent limitations of the data source. Treating NET patients is a complex process. Treatments are individualized based on tumor size, location, and pathology, as well as whether the tumor is functional, type and extent of symptoms, and speed of progression. Our data did not include this level of detail. Insurance companies aggregate information on inpatient and outpatient services (generally reported as ICD-9-CM or ICD-10), procedures (ICD-9 procedure codes and CPT codes) and pharmacy claims (NDC) as claims are submitted for payment by providers, healthcare facilities, and pharmacies. So for example, while GI NET can be identified by using a list of ICD-9-CM codes, presence of advanced disease must be inferred by observing the use of pharmacologic treatment. Another limitation of the data source is that the available length of time for analysis is relatively brief, so conclusions regarding average duration of use may not be representative of long-term treatment patterns.

Our primary finding that 60% of patients initiated pharmacologic therapy with SSA is consistent with the NCCN recommendation of these drugs for initial treatment of clinically significant and progressive NET. Consistent with the clinical observation that SSA tend to be well tolerated, patients initiating treatment with SSA remained on first-line therapy longer than the patients who began treatment with CC and TT. The second finding noted above, that 1/3 of GI NET patients began treatment with CC, is more surprising. CC is relatively ineffective in these patients, and as a result is recommended only if other options are not feasible.

There are several possible explanations for the frequent use of CC. Patients observed to initiate cytotoxic treatment may have been treated in the past with other agents and either progressed or were intolerant to those agents. We reviewed data for 6 mo before the first pharmacologic treatment, but treatments more than 6 mo in the past would have been missed. It may also be that some of the patients had a pathology finding suggesting chemotherapy would be beneficial, or a different type of GI tumor that was incorrectly coded as NET. Our data were de-identified, meaning we could not confirm the diagnosis in medical records, physician or patient surveys, or by other means. Finally, some clinicians may have been unfamiliar with either best practice recommendations or the available, albeit limited, data on GI NET treatment. Our findings are consistent with a recent large case series from a tertiary referral center that found SSA and CC were the two most common treatment strategies used for gastroenteropancreatic NET⁽¹¹⁾.

Previous studies have found significant divergence between clinical guidelines and treatment in other, more common, cancers. Chagpar and colleagues found that while Stage I colon cancer patients were treated according to guidelines by 95%, higher stage patients were less likely to be treated according to guidelines. Stage II

Table 3 Second-line treatment, stratified by first-line treatment *n* (%)

	First-line treatment							Patients with second-line treatment
	SSA	CC	TT	SSA + CC	SSA + TT	TT + CC	IF	
<i>n</i>	128 67.7%	33 17.5%	14 7.4%	5 2.6%	7 3.7%	1 0.5%	1 0.5%	189 100.0%
Second-line treatment								
SSA + TT	51 (39.8)	4 (12.1)	4 (28.6)	3 (60.0)		0 (0)	1 (100.0)	63 (33.3)
SSA + CC	33 (25.8)	6 (18.2)	0 (0)		5 (71.4)	0 (0)	0 (0)	44 (23.3)
CC	24 (18.8)		4 (28.6)	0 (0)	1 (14.3)	0 (0)	0 (0)	29 (15.3)
SSA		16 (48.5)	5 (35.7)	0 (0)	0 (0)	1 (100.0)	0 (0)	22 (11.6)
TT	12 (9.4)	7 (21.2)		2 (40.0)	0 (0)	0 (0)	0 (0)	21 (11.1)
SSA + IF	3 (2.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (1.6)
IF	2 (1.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (1.1)
TT + CC	1 (0.8)	0 (0)	1 (7.1)	0 (0)	0 (0)		0 (0)	2 (1.1)
SSA + TT + CC	1 (0.8)	0 (0)	0 (0)	0 (0)	1 (14.3)	0 (0)	0 (0)	2 (1.1)
SSA + CC + IF	1 (0.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0.5)

SSA: Somatostatin analogues; CC: Cytotoxic chemotherapy; TT: Targeted therapy; IF: Interferon.

high-risk patients showed the lowest concordance with only 36% being treated per recommendation^[12]. In a prospective study of women with breast cancer, Giorano and colleagues found that 83% of patients 55-64 years old received care concordant with chemotherapy guidelines compared with 29% of patients 75 or older^[13].

The finding that only a small proportion of patients are observed to receive second-line treatment is also surprising. The 5-year survival of patients with advanced GI NET is more than 70%. Most surviving patients would be expected to receive continued treatment, whether in the form of liver-directed treatment or pharmacotherapy. We considered multiple explanations for this finding. First, although median follow-up was over 15 mo, many patients were eventually lost to follow-up when they disenrolled from a plan included in our databases. One third of patients were continuing to use their index treatment when they were lost to follow-up. Whether (or when) these individuals progressed to second-line treatment cannot be determined using these databases. If these patients were systematically different from the ones who remained under observation, our results would be biased.

Despite this significant loss to follow-up, nearly 60% of patients were observed to continue enrollment but stop therapy. That is, they survived and remained in the data set, but no second-line pharmacotherapy use could be identified. We considered the possibility that these patients received some liver-directed treatment that alleviated their symptoms or controlled their disease, obviating the need for second-line treatment. However, we found no evidence of this: liver-directed treatment was observed in only 5.5% of patients around the time they stopped first-line treatment. We also considered whether some patients may have had a secondary source of payment, such that their claims for pharmacotherapy did not appear in our databases. Just over 11% of patients were 65

and older and would have been eligible for Medicare. Payment rules regarding patients with both commercial coverage and Medicare are complex^[14] but generally require the commercial payer (for which we did have data) to be primarily responsible for payment. In cases where Medicare had primary responsibility, we would have missed claims for pharmacologic or liver-directed therapy and thus underestimated treatment. The magnitude of this problem is impossible to know using our current data source. A study using Medicare data and examining patients over 65 only might be less likely to suffer from this bias. Finally, it may indeed be the case that some patients stop therapy completely. Such patients may be terminal and choose not to undergo further treatment, or they may be relatively asymptomatic and decline to be treated on that basis. Further research with detailed clinical data would be needed to confirm which, if any, of these explanations is the most accurate.

In this large, claims-based, retrospective study of real-world pharmacologic treatment patterns, we found that 60% of GI NET patients began therapy with SSA and about one-third with CC. The relatively long time to discontinuation of SSA, as well as their use in combination with other agents, suggests they may be well tolerated and potentially have sustained effectiveness. We also found that over half of the patients discontinued treatment after first-line and only less than 10% of the patients received second-line treatment despite the availability of a number of different options. To address the limitations of this study and expand knowledge of real-world treatment patterns, a study using more detailed clinical information such as medical charts or physician surveys is warranted. In addition, future studies should consider using databases that would allow for greater longitudinal follow-up, such as registries, to assist in the further understanding of treatment patterns and length of therapy.

COMMENTS

Background

Neuroendocrine tumors (NET) comprise a broad set of rare tumors. Almost 2/3 arise in the gastrointestinal (GI) tract. The management of GI NET is based on a variety of factors including stage, anatomic location, and the presence and type of symptoms. The most recent NCCN guidelines for unresectable and metastatic GI NET recommend somatostatin analogues (SSA) as first-line treatment, but do not recommend a particular treatment sequence for the remaining therapies.

Research frontiers

This study's results add to the limited knowledge about real-world treatment patterns for GI NET, which is especially significant in light of the lack of treatment guidelines regarding treatment sequences beyond first-line therapy.

Innovations and breakthroughs

This study used two very large, nationally representative claims databases to describe real-world treatment of GI NET. The three key findings were: first, the most common initial pharmacologic treatment was with SSA, with average duration of use of just over 18 mo; second, although 60% of patients initiated treatment with SSA alone or in combination, most of the remainder began treatment with cytotoxic chemotherapy, therapy recommended by NCCN only if no other options (SSA, targeted therapy, or liver directed treatment) are feasible; and third, despite the many available treatment options, less than one in 10 patients was observed to receive treatment with second-line pharmacotherapy of any type. The authors findings are consistent with a recent large case series from a tertiary referral center that found SSA and CC were the two most common treatment strategies used for gastroenteropancreatic NET. Previous studies have also found significant divergence between clinical guidelines and treatment in other, more common, cancers.

Applications

This study suggests that there is frequent use of CC in GI NET treatment, although CC is relatively ineffective in these patients and recommended only if other options are not feasible. This may be a result of clinicians unfamiliarity with either best practice recommendations or the available, albeit limited, data on GI NET treatment. To address the limitations of this study and expand knowledge of real-world treatment patterns, a study using more detailed clinical information such as medical charts or physician surveys is warranted. In addition, future studies should consider using databases that would allow for greater longitudinal follow-up, such as registries, to assist in the further understanding of treatment patterns and length of therapy.

Terminology

NET arise from cells that release hormones in response to nerve stimulation. Insurance claims databases compile coded information related to charges for medical care for large populations, but they do not contain clinically detailed records.

Peer-review

The article aims to describe real-world treatment patterns of GI NET.

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Retrospective Study

Patients with inflammatory bowel disease have increased risk of autoimmune and inflammatory diseases

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Abstract

AIM

To investigate whether immune mediated diseases (IMD) are more frequent in patients with inflammatory bowel disease (IBD).

METHODS

In this population based registry study, a total of 47325 patients with IBD were alive and registered in the Danish National Patient Registry on December 16, 2013. Controls were randomly selected from the Danish Civil Registration System (CRS) and matched for sex, age, and municipality. We used ICD 10 codes to identify the diagnoses of the included patients. The IBD population was divided into three subgroups: Ulcerative colitis (UC), Crohn's disease (CD) and Both the latter referring to those registered with both diagnoses. Subsequently, odds-ratios (OR) and 95%CI were obtained separately for each group and their respective controls. The use of Bonferoni post-test correction adjusted the significance level to $P < 0.00125$. P -values were estimated using Fisher's exact test.

RESULTS

There were significantly more women than men in the registry, and a greater percentage of comorbidity in the IBD groups ($P < 0.05$). Twenty different IMDs were all significantly more frequent in the IBD group. Sixteen

were associated with UC versus twelve with CD. In both UC and CD ORs were significantly increased ($P < 0.00125$) for primary sclerosing cholangitis (PSC), celiac disease, type 1 diabetes (T1D), sarcoidosis, asthma, iridocyclitis, psoriasis, pyoderma gangrenosum, rheumatoid arthritis, and ankylosing spondylitis. Restricted to UC ($P < 0.00125$) were autoimmune hepatitis, primary biliary cholangitis, Grave's disease, polymyalgia rheumatica, temporal arteritis, and atrophic gastritis. Restricted to CD ($P < 0.00125$) were psoriatic arthritis and episcleritis. Restricted to women with UC ($P < 0.00125$) were atrophic gastritis, rheumatoid arthritis, temporal arteritis, and polymyalgia rheumatica. Restricted to women with CD were episcleritis, rheumatoid arthritis, and psoriatic arthritis. The only disease restricted to men ($P < 0.00125$) was sarcoidosis.

CONCLUSION

Immune mediated diseases were significantly more frequent in patients with IBD. Our results strengthen the hypothesis that some IMDs and IBD may have overlapping pathogenic pathways.

Key words: Immune mediated diseases; Ulcerative colitis; Risk; Prevalence; Registry; Chronic inflammatory diseases; Autoimmune diseases; Inflammatory bowel disease; Crohn's disease; Extraintestinal manifestations

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Core tip: Essential to inflammatory bowel disease (IBD) pathogenesis are environmental factors, altered gut microbiota and genetic susceptibility. The latter causing impairment of barrier function, autophagy, and Th1, 2 and 17 cell responses. Interestingly, these mechanisms are also thought important in other immune mediated diseases, as is the overlap of susceptibility genes. Besides the classic extraintestinal manifestations, we found a variety of immune mediated diseases to be more frequent in individuals with IBD. Physicians should be aware of this when treating these patients. Furthermore, these findings support the hypothesis that immune mediated diseases may have overlapping pathogenesises. Thus, understanding IBD might help us understand other immune mediated diseases and vice versa.

Halling ML, Kjeldsen J, Knudsen T, Nielsen J, Koch Hansen L. Patients with inflammatory bowel disease have increased risk of autoimmune and inflammatory diseases. *World J Gastroenterol* 2017; 23(33): 6137-6146 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6137.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6137>

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are two

distinct types of chronic inflammatory bowel diseases. The insight into etiology factors and the complex pathogenetic process is not yet fully understood. The diseases are often diagnosed in young individuals and recent studies report increasing incidences of both UC and CD, not only in Denmark but globally^[1]. It has been suggested that inflammatory bowel disease (IBD) may be due to an inappropriate inflammatory response to the intestinal flora in genetically susceptible individuals. So far, several susceptibility genes have been identified^[2]. Many of these are also found in other immune mediated diseases (IMDs), indicating overlaps between pathogenic pathways. The identified risk genes in IBD are involved in maintaining normal microbial gut homeostasis and adequate immune response^[3,4]. Mutations in these may impair mechanisms essential to innate and adaptive immune response, *i.e.* weakened mucosal barrier, a decrease of antibacterial agents, impaired autophagy and antigen recognition. Mutations may also cause an imbalance of pro- and anti-inflammatory cytokines related to the regulation of Th1, 2 and 17 in particular^[5]. CD is considered Th1 mediated thus characterized by interferon gamma, tumor necrosis factor alpha, and IL 12. UC is associated with a Th2 response where IL 4, 5, 10 and 13 are dominant. The Th17 response is present in both CD and UC but most pronounced in CD. It is characterized by IL 17 and 23 production. Th17 can also produce interferon gamma like Th1^[5-8]. It is suggested that disturbances in these mechanisms may cause a loss of self-tolerance leading towards chronic inflammation or autoimmunity^[9-12].

The gut microbiota of patients with IBD has been shown to contain less diversity, a reduced number of bacteria, and an altered microbial metabolite profile compared to healthy individuals^[13]. Environmental factors, *i.e.* medication (antibiotics, non-steroid anti-inflammatory drugs and hormones), diet, geography, and previous infections might influence this^[4]. A similar etiology is believed to exist in other IMDs, *i.e.* rheumatoid arthritis, ankylosing spondylitis, multiple sclerosis, type 1 diabetes (T1D), and celiac disease^[14,15].

It has become clear, that patients with an existing IMD are more likely to develop other IMDs, this is more evident in females than in males^[16]. Apart from the extraintestinal manifestations of IBD, little is known about the association between IBD and other IMDs.

Only a few large population based studies on the subject exist. The results of these suggest that IBD is associated with asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, autoimmune thyroiditis, T1D, and vasculitis^[17-22]. Different study designs, varying validity of diagnoses, population sizes and confounders, *i.e.* ethnicity, economic and social status, all make the findings of these studies difficult to interpret.

In Denmark healthcare is free and all contacts to hospitals are registered on an individual basis based on a civil registration number together with diagnosis

Table 1 Participants' demographic data

Variables	IBD	Control	UC	Control	CD	Control	Both ¹	Control
<i>n</i>	47325	92839	31066	60951	13343	26172	2916	5716
Female	54%	55%	53%	53%	58%	58%	56%	56%
Male	46%	45%	47%	47%	42%	42%	44%	44%
Mean age at entry, yr	53	53	55	55	49	49	47	47
Mean age at onset of IBD, yr	42	-	44	-	37	-	34	-
Mean duration of IBD at entry, yr	10	-	9	-	10	-	11	-
Comorbidity 0 ²	77%	83%	76.50%	82.00%	77%	85%	82%	87%
Comorbidity 1-2	18%	13.50%	18.00%	14%	18%	12%	15%	10%
Comorbidity ≥ 3	5%	3.50%	5.50%	4%	5%	3%	3%	3%

¹Patients registered with both CD and UC; ²No. of comorbidities at onset of IBD according to the Charlson comorbidity index. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

and procedural codes. This allows a unique access to information not confounded by economic and social status.

The aim of this study was to examine if IMDs are more frequent among patients with CD and UC compared to the background population.

MATERIALS AND METHODS

This was a cross-sectional study including all living patients with IBD who were matched with a control group to compare the point-prevalence of specific IMDs.

Identification of patients and controls

The Danish National Patient Registry include all contacts within the healthcare system both in-hospital, since 1977, and in outpatient settings since 1994. Data were retrieved on December 16, 2013 and included all patients alive registered with a diagnosis compatible with CD and UC. Patients were identified using the ICD 10 codes: CD K50.0-K50.9; UC, K51.0-K51.9). ICD 10 codes including "other" or "unspecified" were excluded to avoid inclusion of non-specific diseases and incorrect diagnosis codes.

The Danish Civil Registration System (CRS) includes all Danish inhabitants and each person has a unique 10-digit identification number. The CRS includes demographic data *e.g.* name, sex, date of birth, and death^[23]. All IBD patients were paired (2:1) with random controls identified in the CRS and matched by sex, age (± 1 year) and municipality. Demographic data presented are based on data from the CRS.

The selected forty IMDs are all considered to be of either autoimmune or inflammatory origin. The same criteria were used for the IMDs. ICD 10 codes for the IMDs are listed in the Supplementary Table 1.

To assess comorbidity we used the Charlson comorbidity index which has been developed to estimate 1-year mortality in cancer patients. It is also useful in research to identify possible confounding diseases. It includes a number of systemic diseases associated with increased mortality, *i.e.* organ failure,

AIDS, and cancer^[24].

Ethics

This study was approved by the Danish Data Protection Agency (approval # 2013-41-1596). Approval from the Ethics Committee was not needed as this is a registry study.

Statistical analysis

The occurrence of IMDs was obtained separately for each group. Then OR and 95%CI were calculated. Fisher's exact test was used to calculate *P*-values.

We used the Bonferroni post-test correction to reduce the likelihood of false positives. We did 40 comparisons (the 40 IMDs investigated) and adjusted the significance level accordingly to $P < 0.00125$. Calculations was made using STATA version 13.0 (StataCorp LP, TX, United States).

RESULTS

A total of 47325 patients were alive and registered with IBD on December 16, 2013. A total of 92839 controls were identified.

CD was registered in 13343 patients, UC in 31066, and 2916 were registered with both diagnoses. A total of 92839 controls were found for the IBD group, 26172 for CD, 60951 for UC and 5716 for those with both diagnoses. Due to the matching criteria, five IBD patients had only one or no controls.

There was an excess of women in all IBD groups, most pronounced in CD ($P < 0.05$). The mean age at onset of disease was significantly higher in UC. Comorbidity was most frequent in those with either UC or CD ($P < 0.05$). See Table 1.

Twenty out of forty IMDs had significantly increased ORs in the IBD groups compared to their controls ($P < 0.00125$). Sixteen IMDs were associated with UC and twelve with CD. See Tables 2 and 3.

Seven of the IMDs were considered rheumatologic diseases, included ankylosing spondylitis, rheumatoid arthritis, psoriatic arthritis, polymyalgia rheumatic, temporal arteritis, polyarteritis nodosa, and Churg

Table 2 Number of immune mediated diseases

Disease	IBD	Control	CD	Control	UC	Control	Both ¹	Control
Primary sclerosing cholangitis	257	4	35	1	192	2	30	1
Pyoderma gangrenosum	193	8	60	1	97	7	36	0
Autoimmune hepatitis	124	35	15	11	96	22	13	2
Celiac disease	280	92	133	30	132	58	15	4
Ankylosing spondylitis	431	151	189	32	201	102	41	17
Churg Strauss syndrome	14	5	4	1	8	4	2	0
Primary biliary cholangitis	71	32	11	6	53	25	7	1
Episcleritis	56	33	25	9	23	21	8	3
Iridocyclitis	419	295	148	82	230	188	41	25
Atrophic gastritis	60	47	16	11	42	34	2	2
Psoriasis	378	345	148	99	200	229	30	17
Polyarteritis nodosa	42	38	15	9	24	27	3	2
Rheumatoid arthritis	446	401	119	110	250	311	32	25
Type 1 diabetes	1682	1464	359	431	1002	1180	103	71
Sarcoidosis	141	122	29	38	79	94	14	9
Asthma	1140	981	337	363	568	695	76	82
Giant cell arteritis	193	156	37	46	116	141	3	6
Psoriatic arthritis	316	249	81	93	147	206	21	17
Grave's disease	817	581	141	207	394	561	46	49
Polymyalgia rheumatica	468	320	72	122	242	324	6	22

¹Patients registered with both CD and UC. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Table 3 Odds-ratios for immune mediated diseases, in patients with inflammatory bowel disease

Disease	IBD	95%CI	UC	95%CI	CD	95%CI	Both ¹	95%CI
Primary sclerosing cholangitis	126.7 ^a	47.2-340.3	189.5 ^a	47.0-763.4	68.8 ^a	9.4-502.6	59.4 ^a	8.1-436.2
Pyoderma gangrenosum	47.5 ^a	23.4-96.4	27.3 ^a	12.7-58.7	118.2 ^a	16.4-853.3	36/0 ^{a,2}	
Autoimmune hepatitis	7.0 ^a	4.8-10.1	8.6 ^a	5.4-13.6	2.7 ^b	1.2-5.8	12.8 ^a	2.9-56.8
Celiac disease	6.0 ^a	4.7-7.6	4.5 ^a	3.3-6.1	8.8 ^a	5.9-13.0	7.4 ^a	2.4-22.3
Ankylosing spondylitis	5.6 ^a	4.7-6.8	3.9 ^a	3.1-4.9	11.7 ^a	8.1-17.1	4.8 ^a	2.7-8.4
Churg Strauss syndrome	5.5 ^a	2.0-15.3	3.9 ^b	1.2-13.0	- ^c		- ^c	
Primary biliary cholangitis	4.4 ^a	2.9-6.6	4.2 ^a	2.6-6.7	3.6 ^b	1.3-9.7	13.8 ^b	1.7-111.9
Episcleritis	3.3 ^a	2.2-5.1	2.1 ^b	1.2-3.9	5.5 ^a	2.5-11.7	5.2 ^b	1.4-19.8
Iridocyclitis	2.8 ^a	2.4-3.3	2.4 ^a	2.0-2.9	3.6 ^a	2.7-4.7	3.2 ^a	2.0-5.4
Atrophic gastritis	2.5 ^a	1.7-3.7	2.4 ^a	1.5-3.8	2.9 ^b	1.3-6.2	- ^c	
Psoriasis	2.2 ^a	1.9-2.5	1.7 ^a	1.4-2.1	3.0 ^a	2.3-3.8	3.5 ^a	1.9-6.5
Polyarteritis nodosa	2.2 ^a	1.4-3.4	1.7 ^b	1.0-3.0	3.3 ^b	1.4-7.5	- ^c	
Rheumatoid arthritis	1.8 ^a	1.5-2.0	1.6 ^a	1.3-1.9	2.1 ^a	1.6-2.8	2.5 ^a	1.5-4.2
Type 1 diabetes	1.7 ^a	1.6-1.9	1.7 ^a	1.6-1.8	1.7 ^a	1.4-1.9	2.9 ^a	2.2-3.9
Sarcoidosis	1.7 ^a	1.3-2.2	1.7 ^a	1.2-2.2	- ^c		3.1 ^b	1.9-4.8
Asthma	1.7 ^a	1.6-1.9	1.6 ^a	1.4-1.8	1.8 ^a	1.6-2.1	1.8 ^a	1.3-2.5
Giant cell arteritis	1.6 ^a	1.3-2.0	1.6 ^a	1.3-2.1	1.6 ^b	1.0-2.4	- ^c	
Psoriatic arthritis	1.5 ^a	1.3-1.8	1.4 ^b	1.1-1.7	1.7 ^a	1.3-2.3	2.4 ^b	1.3-4.6
Grave's disease	1.4 ^a	1.3-1.6	1.4 ^a	1.2-1.6	1.3 ^b	1.1-1.7	1.9 ^b	1.2-2.8
Polymyalgia rheumatica	1.3 ^a	1.2-1.5	1.5 ^a	1.2-1.7	- ^c		- ^c	

^a $P < 0.00125$; ^b $P = 0.00125-0.05$; ^c $P > 0.05$; ¹Patients registered with both CD and UC; ²No. of cases in IBD cohort/No. of cases in control cohort. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

Strauss Syndrome.

Five IMDs were gastrointestinal including celiac disease, atrophic gastritis, primary sclerosing cholangitis, primary biliary cholangitis, and autoimmune hepatitis.

The remaining IMDs were T1D, Grave's disease, pyoderma gangrenosum, psoriasis, iridocyclitis, episcleritis, sarcoidosis, and asthma.

There was a trend towards significance ($P = 0.00125-0.05$) for Wegener's granulomatosis, chorioretinitis, vitiligo, lichen ruber planus, scleroderma, and multiple sclerosis.

Seven IMDs were only significant in women. While only one was restricted to men. See Table 4.

In general, the same pattern is seen in those registered with both CD and UC.

We did not observe any OR below one, neither did we record any cases of Sjögren's syndrome, inclusion body myositis, eosinophilic esophagitis, or autoimmune adrenalitis.

DISCUSSION

In this study, we documented an increased frequency

Table 4 Odds-ratios for immune mediated diseases restricted to either gender

Disease	Females	95%CI	Males	95%CI
IBD				
Episcleritis	3.6 ^a	2.1-6.1	2.9 ^b	1.4-6.1
Atrophic gastritis	3.5 ^a	2.1-5.9	- ^c	
Polyarteritis nodosa	2.6 ^a	1.5-4.5	- ^c	
Rheumatoid arthritis	1.9 ^a	1.6-2.2	1.4 ^b	1.1-1.9
Giant cell arteritis	1.7 ^a	1.3-2.2	- ^c	
Psoriatic arthritis	1.6 ^a	1.3-2.0	1.4 ^b	1.1-1.9
Polymyalgia rheumatica	1.5 ^a	1.3-1.8	- ^c	
Sarcoidosis	1.5 ^b	1.1-2.2	1.9 ^a	1.3-2.6
UC				
Atrophic gastritis	3.1 ^a	1.7-5.8	- ^c	
Rheumatoid arthritis	1.7 ^a	1.4-2.1	- ^c	
Giant cell arteritis	1.7 ^a	1.3-2.3	- ^c	
Polymyalgia rheumatica	1.6 ^a	1.3-2.0	- ^c	
CD				
Episcleritis	5.9 ^a	2.4-15.0	4.5 ^b	1.2-17.5
Rheumatoid arthritis	2.3 ^a	1.7-3.0	- ^c	
Psoriatic arthritis	2.0 ^a	1.3-2.8	- ^c	
Sarcoidosis	- ^c		3.2 ^a	1.6-6.6
Both ¹				
Iridocyclitis	3.6 ^a	1.9-6.8	2.7	1.2-6.2
Celiac disease	6.0 ^a	1.9-18.6	3/0 ^{b,2}	
Autoimmune hepatitis	17.9 ^a	2.3-141.5	7.8 ^b	0.9-69.8

^aP < 0.00125; ^bP = 0.0125-0.05; ^cP > 0.05; ¹Patients registered with both CD and UC; ²No. of cases in IBD cohort/No. of cases in control cohort. CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease.

of twenty IMDs in patients with IBD compared to matched cohorts.

Although most of the IMDs are considered to be Th1 mediated, UC was associated with more IMDs than CD. The presence of Th17 cells in UC and their ability to induce a Th1 response might explain this. Another explanation might be that certain susceptibility genes can act differently depending on the setting^[25]. A gene might increase the risk of one disease while reducing the risk of others^[25-27].

Extraintestinal manifestations

Ankylosing spondylitis, pyoderma gangrenosum, psoriasis, iridocyclitis, episcleritis, and primary sclerosing cholangitis (PSC) are all well described in IBD^[28]. Thus the significant associations were expected. Except from PSC, these will not be discussed further.

Primary sclerosing cholangitis and gastrointestinal immune mediated diseases

PSC is predominant in men and most frequent in UC^[29]. We found PSC to be associated with both types of IBD and both genders. Most striking is the association with CD which is less often described. Studies suggest that PSC is more frequent when colon is affected and a distinct subtype, PSC-IBD has been suggested^[30-33]. This study does not include data on localization, severity or extension. Several PSC risk genes are shared with IBD and other IMDs^[33,34]. Gene mutations influencing IL 10 signaling are identified in CD, UC

and PSC. The absence of IL 10 can cause severe CD due to lack of Th1 and macrophage inhibition^[33-35]. Interestingly, hepatobiliary inflammation is thought to be induced by microbial metabolites and changes in the microbiota and this inflammation is linked to the *FUT2* gene, which is also found in CD^[33,34,36].

In contrast to most other studies^[18,36,37], we found celiac disease to be more frequent in those with IBD regardless of type, as did another Danish study^[16]. Other studies found IBD to be more common in patients with celiac disease but not vice versa^[38-40]. Similarities and differences in pathogenesis might explain these conflicting results. Celiac disease is like IBD an inflammatory disorder of the intestine, often diagnosed in young individuals, more common in women, and Th1 mediated. Changes in microbiota and dysfunctional IL 18 receptor are also noted in both conditions^[27,41]. Risk genes of celiac disease shared with CD relates to adaptive immunity while those shared with UC primarily relates to barrier function. Different from IBD is the absence of Th17 response, impaired autophagy and while important in celiac disease, IL 15 is not that important in IBD^[41].

We found autoimmune hepatitis, primary biliary cholangitis, and atrophic gastritis to be more common in UC only. Again, results from previous studies conflict^[16,18,21,42-45]. Little is known about the association with atrophic gastritis, which to our knowledge is unique to this study. Th1, 2 and 17 responses are important in IBD, PSC and primary biliary cholangitis pathogenesis. Primary biliary cholangitis and IBD have overlapping susceptibility genes, which is not the case with autoimmune hepatitis^[46,47]. The pathogenesis of primary biliary cholangitis resembles those of autoimmune hepatitis and CD, dysfunctions in IL 12 signaling promotes a Th1 and possibly also a Th17 response, causing a granulomatous inflammation^[47,48].

Endocrine diseases

UC is reported to occur more frequently in family members of patients with T1D^[18,19,49]. However, three studies did not find any association^[16,20,21]. This study found T1D associated with both UC and CD. Confounding due to treatment with corticosteroids is unlikely, as the mechanisms in steroid induced diabetes resemble those in type 2 diabetes^[50,51]. Levels of IL 18 are elevated in CD and T1D, but not in UC. IL 18 causes a Th1 response and is likely to affect mucosal barrier function too^[27,52]. PTPN2 is one of many shared risk genes^[2,53]. It promotes beta cell apoptosis in T1D while causing intestinal barrier dysfunction, impaired autophagocytosis, and inhibition of Th17 in IBD^[25,54]. Changes in the gut microbiota are also suggested to trigger T1D^[27].

Data on autoimmune thyroiditis and IBD is sparse, similarities to IBD limited and only few risk genes overlap^[55-57]. Restricted to UC only, we found OR significantly increased for Grave's disease. None was

detected for Hashimoto's thyroiditis. Similar results are reported in two other studies^[18,58]. One study reports hypothyroidism more common in CD^[19]. In addition, three other studies did not find any association at all^[16,17,21].

Rheumatic diseases

Rheumatoid arthritis was associated with both UC and CD while psoriatic arthritis was restricted to CD. Previously published data support this^[18,20,21,59]. The microbiome of the gut and skin are possible triggers in rheumatoid arthritis and psoriatic arthritis^[60]. Both types of arthritis share characteristics with CD in particular. Th1 and 17 are essential in all three pathogeneses^[2,61-64].

ORs for polymyalgia rheumatica and temporal arteritis were significantly increased in the IBD and UC group, not in CD. This is supported by one study while refuted by another^[16,18]. Overlapping susceptibility genes suggest that Th1, Th17 and regulatory T cells are of importance to the pathogeneses^[65].

ORs for Churg Strauss Syndrome and polyarteritis nodosa were significantly increased in the overall IBD group but not in the subgroups. The low number of cases calls for careful interpretation and future studies.

Other disorders

In this study, asthma was more common in both UC and CD. Both UC and allergic asthma are considered Th2 mediated. Also, a Th17 response is described in severe asthma^[66]. Risk genes are associated with IL 13 and 17 production, dysfunctional regulatory T cells and regulation of Th1, 2 and 17 responses^[26,66]. Studies have not found that asthma reduces the risk of IBD^[67,68], rather the opposite seems more likely^[17,18,20,21].

The association of sarcoidosis and IBD were restricted to UC and males with CD. Another study confirms the linkage to UC^[18]. There is not much documentation for this association. The inflammation in sarcoidosis is similar to CD; granulomatous; Th1 and 17 driven; and mutations in NOD2 and IL 23 receptor gene are identified^[2,69-71].

There were no cases of Sjögren's syndrome, inclusion body myositis, eosinophilic esophagitis, or autoimmune adrenalitis. This is unexpected. Some case reports have described the coexistence of Sjögren and primary adrenocortical insufficiency in IBD patients^[16,72-74]. One case report describes eosinophilic esophagitis and CD^[75]. While to our knowledge, no association between inclusion myositis and IBD has been reported. Although specific ICD 10 codes were used misclassification is still possible e.g. autoimmune adrenalitis might be registered as Addison's disease.

Strengths and limitations

The strength of this study is that it includes all patients alive with CD or UC in Denmark. The Danish

population is homogenous regarding ethnicity and religion. Health care is free to all residents; thus, NPR is not biased by inclusion of specific hospitals, age groups, insurance policies, social, or financial status. As the general practitioners do not provide data, diseases not requiring hospital treatment could be underrepresented i.e. asthma, Grave's disease, Hashimoto's thyroiditis, and atrophic gastritis^[76].

A limitation of the study is possible bias caused by varying validity of the ICD 10 codes. Only few Danish studies have addressed this issue. The average positive predictive value (PPV) of an ICD 10 diagnosis for any medical condition in the NPR varies from 65.5 % to 81%^[76].

However, the completeness is 94% for both UC and CD while the PPV of UC and CD is 90% and 97% respectively^[77].

The validity of T1D is like that of IBD, very high^[24,78]. The PPV of asthma among hospitalized children is 85% while 65% among adults. However, a sensitivity analysis did not find the PPV in adults, low enough to nullify the hypothesis^[79,80]. As a collective group the PPV of connective tissue diseases is reported as high^[24]. The PPV of rheumatoid arthritis is low^[81].

Despite varying validity of ICD 10 codes, most of our findings are in alignment with those of the studies using algorithms to increase the validity. Important to this study, is the occurrence of the classic extraintestinal manifestations which indicates that our results are not too biased.

Detection bias is another concern. Patients seen on regular basis by a physician such as those with IBD are more likely to be diagnosed.

To eliminate confounders like sex, age and geography in the IBD group, we used these as matching criteria. Information regarding smoking status was not available to us, thus no correction was made.

Another confounder is drug induced autoimmunity. A wide variety of drugs are suggested to induce autoimmunity. Among these are antibiotics, statins, methotrexate, thiopurines, and biological agents (anti-TNF- α agents)^[82-88]. Biological agents, which are often used to treat IBD, ankylosing spondylitis, psoriasis, and rheumatoid arthritis, are paradoxically suggested to induce IMDs. No correction was made since we do not have data regarding patients' use of prescribed drugs.

While Bonferroni post-test correction reduced the risk of false positives, the risk of false negatives simultaneously increased. Knowing this, a low number of false positives were still preferred in this study.

In conclusion, our study emphasizes that immune mediated diseases are more frequent among patients with CD or UC. Our results strengthen the thesis of partially overlapping pathogeneses among some immune mediated diseases including IBD and emphasized the complexity of IBD pathogenesis. Our most important findings are the increased risk of

celiac disease and T1D in both UC and CD, but also the increased risk of primary sclerosing cholangitis in CD although not being limited to CD. Finally, when treating patients with UC or CD one should be aware of the strong association with other immune mediated diseases.

COMMENTS

Background

Extraintestinal manifestations in Crohn's disease (CD) and ulcerative colitis (UC) are well described. The authors aimed to investigate whether other immune mediated diseases were associated with inflammatory bowel disease (IBD).

Research frontiers

Most studies on the subject are small or case reports. Only few larger studies have been conducted. The authors aimed to estimate odds-ratios of developing an immune mediated diseases (IMD) in patients with IBD compared individuals without IBD.

Innovations and breakthroughs

This is one of few larger studies on the subject. It includes all patients alive with CD or UC in Denmark. Due to free health care to all residents the study is unbiased by inclusion of specific hospitals, age groups, insurance policies, social or financial status. The authors found several IMDs not considered classic extraintestinal manifestations to be significantly associated with IBD.

Applications

Physicians treating patients with IBD should be aware of the increased risk of developing other IMDs than the classic extraintestinal manifestations. The findings support the hypothesis that shared pathogenic pathways among IMDs could exist.

Peer-review

It's a well-written and interesting manuscript.

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Retrospective Study

Suspicious brush cytology is an indication for liver transplantation evaluation in primary sclerosing cholangitis

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Abstract

AIM

To investigate markers for high-grade dysplasia for the optimal timing of liver transplantation in patients with primary sclerosing cholangitis (PSC).

METHODS

Earlier data support a dysplasia-carcinoma sequence, even low- to high-grade dysplasia, in PSC-associated cholangiocarcinoma (CCA). Surveillance using endoscopic retrograde cholangiography (ERC) and brush cytology aims to detect cases of biliary dysplasia, and liver transplantation is an option in cases with suspicion of malignancy in brushing. This study investigated markers to identify patients with high-grade biliary dysplasia for optimal timing in early liver transplantation. Patients undergoing surveillance using ERC and brush cytology during 2008-2014 and who were diagnosed with biliary dysplasia in explanted liver or CCA until February 2016 were included in the study. Demographic data, cholangiography findings, laboratory values, cytological morphology and DNA ploidy were

analysed.

RESULTS

Thirty PSC patients had biliary neoplasia in the explanted liver during the study period. Sixteen of these patients had low-grade dysplasia, 10 patients had high-grade dysplasia, and 4 patients had CCA. Fifteen PSC patients diagnosed with CCA were not transplanted. Patients with low-grade dysplasia were younger. Alkaline phosphatase or carcinoembryonic antigen values did not differ between groups during surveillance, but carbohydrate antigen 19-9 was higher in CCA patients. No difference in PSC duration, ERC scores, suspicious cytology, or ploidy analysis was found between groups. No difference was observed between fibrosis stage in explanted livers. Low- and high-grade dysplasia could not be differentiated before liver transplantation based on liver enzymes, tumour markers, ERC scores, brush cytology or DNA ploidy.

CONCLUSION

Repeated suspicion of neoplasia in brush cytology should be an indication for evaluations of liver transplantation prior to the development of CCA.

Key words: Endoscopic retrograde cholangiography; Brush cytology; Cholangiocarcinoma; Biliary dysplasia

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Core tip: We investigated markers of high-grade dysplasia for the optimal timing of early liver transplantation (LT) in patients with primary sclerosing cholangitis (PSC). PSC patients in our unit undergo surveillance with endoscopic retrograde cholangiography (ERC) and brush cytology (BC) to identify evidence of dysplasia before progression to cholangiocarcinoma. Carbohydrate antigen 19-9 was higher in patients with cholangiocarcinoma, but no other differences between laboratory values, ERC scores, BC or ploidy analysis between the low-grade, high-grade or CCA groups were observed. Repeated suspicion of neoplasia in BC should be an indication for the evaluation for LT prior to the development of cholangiocarcinoma.

Boyd S, Vannas M, Jokelainen K, Isoniemi H, Mäkisalo H, Färkkilä MA, Arola J. Suspicious brush cytology is an indication for liver transplantation evaluation in primary sclerosing cholangitis. *World J Gastroenterol* 2017; 23(33): 6147-6154 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6147.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6147>

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease that often presents in conjunction with

inflammatory bowel disease (IBD). PSC leads to bile duct strictures and liver fibrosis. PSC also markedly increases the risk for cholangiocarcinoma (CCA), with a lifetime risk of 5%-10%^[1,2]. Bile duct dysplasia is a precursor for CCA^[3] in PSC. Brush cytology (BC) and imaging are used for the detection of malignancy in PSC.

The incidence of PSC is high in Nordic countries. The primary diagnosis of PSC in our hospital uses endoscopic retrograde cholangiography (ERC) with BC in all patients for individual risk stratification to estimate disease progression and risk for dysplasia^[4]. PSC patients also undergo a regular surveillance programme using ERC and BC according to a previously described protocol^[5], which demonstrated that the frequency of ERCs depends on disease severity in ERC and earlier results in BC and ploidy analysis. The indications for liver transplantation (LT) in PSC patients include end-stage liver disease or symptoms of the disease (e.g., recurrent cholangitis). CCA is also an indication for LT in combination with chemoradiation in select centres^[6]. CCA is generally a contraindication for LT in our centre. However, LT may be considered to prevent progression to CCA^[4,7] in cases with biliary dysplasia.

This study evaluated risk factors in PSC patients with histologically confirmed biliary dysplasia or CCA.

MATERIALS AND METHODS

Patients

This study included PSC patients with verified biliary dysplasia or CCA. There were 588 PSC patients under surveillance at the Helsinki University Hospital during 2008-2014. PSC patients in the study population who were diagnosed with CCA or biliary dysplasia until February 2016 were included in this study. All explanted PSC livers were re-evaluated, and cases with biliary dysplasia or CCA ($n = 30$) were included. All PSC patients who were diagnosed with CCA during the aforementioned period who did not undergo liver transplantation were included ($n = 15$). At least one ERC was performed for each patient during the study period. Low-grade dysplasia (LGD) of the bile ducts was observed in 16 transplanted patients, and high-grade dysplasia (HGD), including carcinoma in situ, was observed in 10 patients with LT. CCA was observed in four patients with LT and 15 patients without LT. Suspicion of biliary dysplasia was considered an important decision criterion for evaluation for liver transplantation in 7/10 patients with HGD, 12/16 patients with LGD, and 4/4 patients with CCA. Thirty-two patients (71%) were male, and 13 patients were female.

Laboratory results

Laboratory parameters [carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), alkaline

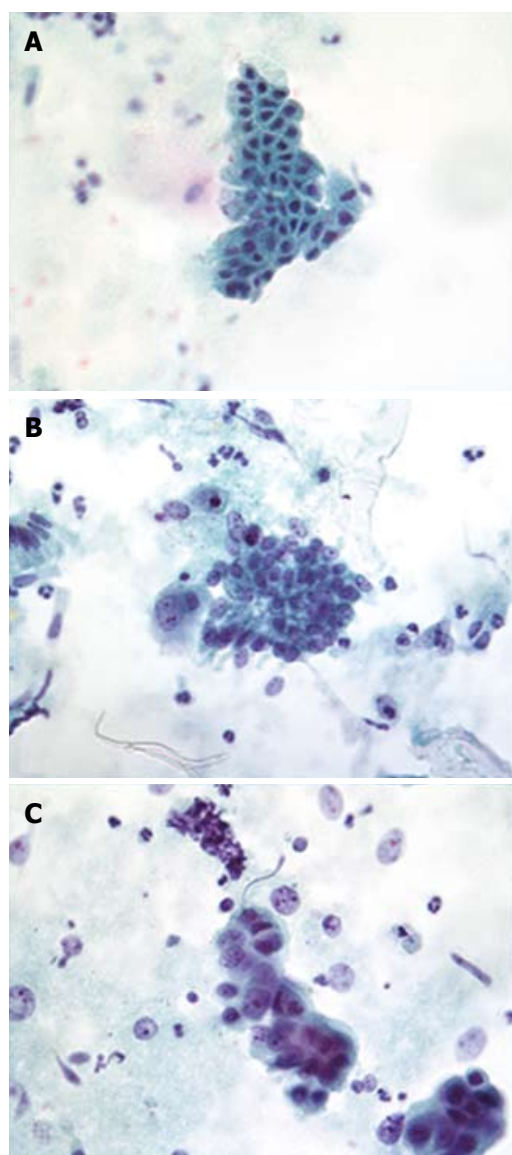


Figure 1 Representative examples of bile duct brushings. A: Benign brush cytology with inflammatory atypia; B: Brush cytology with suspicion of malignancy; C: Malignant brush cytology. Patient was diagnosed with cholangiocarcinoma.

phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine, bilirubin, and international normalised ratio (INR)] were collected one day before the latest ERC.

Imaging studies

Images from the latest ERC were scored according to a modified Amsterdam score^[4]. An MRI and/or CT were performed prior to LT.

Brush cytology and ploidy analysis

All brush samples and DNA flow cytometry specimens until LT or detection of CCA were included. Brush samples were re-evaluated and graded as benign (including atypical due to inflammation or regeneration), suspicious for neoplasia, or malignant (Figure 1).

Table 1 Histological findings in 30 explanted livers of primary sclerosing cholangitis patients with biliary low-grade dysplasia or high-grade dysplasia/cholangiocarcinoma

	LGD	HGD + CCA ²	P value
<i>n</i>	16	14	
Early LT ¹ , <i>n</i>	12	11	1.000
Histology intrahepatic			
Liver weight, g (IQR)	1380 (1190-1717)	1408 (1262-1487)	0.910
Fibrosis stage 1-4 (IQR)	3.5 (3.0-4.0)	3.0 (2.0-4.0)	0.473
Stage 1-2, <i>n</i>	3	4	0.675
Stage 3-4, <i>n</i>	13	10	0.675
Histology extrahepatic, <i>n</i>			
Purulent cholangitis	12	6	0.142
Ulceration	11	6	0.224
Intestinal metaplasia	2	1	0.822
Squamous metaplasia	1	0	0.790

¹In early LTs, suspicion of biliary neoplasia was an important decision criterion for LT; ²CCA four patients; HGD ten patients. Values are median values unless otherwise indicated (IQR). *P* values: Mann-Whitney *U*-test for continuous variables, Fisher's exact test for dichotomous variables. CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile range; LGD: Low-grade dysplasia; LT: Liver transplantation.

Cytocentrifuge slides and slides from cellblocks were evaluated when cellblocks were available. Ploidy analyses were performed as previously described^[7]. Briefly, a separate sample for DNA flow cytometry was obtained before the BC sample and added to RPMI 1640 medium (Gibco/Thermo Scientific, MA, United States) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 0.5% heparin and 0.5% human serum albumin. Cells were centrifuged, washed, treated with RNase (Sigma Chemical Company, MO, USA) and stained with ethidium bromide (Sigma Chemical Company, MO, United States) in Tris-EDTA buffer. HL-60 cells were used as a normal control, and goose and salmon trout red blood cells were used as internal controls. DNA flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences, Oxford, United Kingdom). The results were analysed using ModFit LT software (Becton Dickinson Immunocytometry Systems, Becton, Dickinson and Company, Franklin Lakes, NJ, United States), and a DNA index over 1.1 was considered aneuploidy.

Histology

Two pathologists, who were blinded to the BC or original interpretation, re-evaluated explanted livers and bile ducts or other histological samples (Table 1, Figure 2). Biliary dysplasia was graded as high-grade or low-grade. Patients were divided into groups (LGD, HGD, CCA) using the worst histological finding.

Statistical analysis

Statistical analyses were performed using the IBM SPSS statistical software package version 24 (IBM, New York, NY, United States). The Jonckheere-Terpstra and Mann-Whitney *U*-tests were used to evaluate

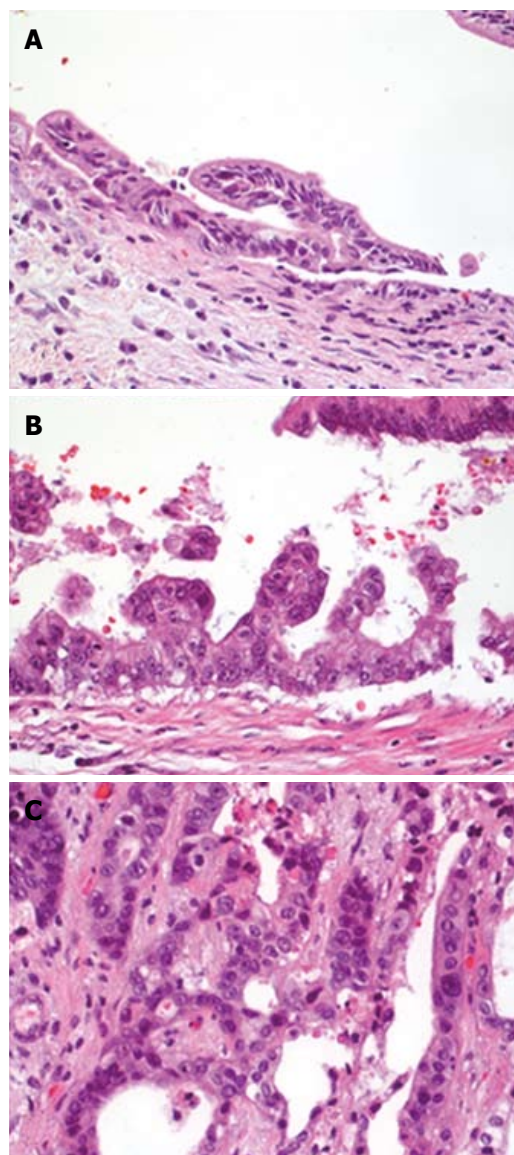


Figure 2 Histology from explanted livers diagnosed with biliary neoplasia. A: Bile duct from explanted liver with low-grade dysplasia; B: Bile duct from explanted liver with high-grade dysplasia; C: Cholangiocarcinoma from explanted liver.

differences in continuous variables between groups. Fisher's exact test was used for dichotomous variables. Bonferroni correction was used in pairwise tests, and adjusted values are presented.

Ethics

The local ethics committee approved the study protocol.

RESULTS

Demographics

There was no statistically significant gender difference between groups (Table 2), but all groups included a majority of male patients (32 males and 13 females in total). Patients with LGD were significantly younger than other patients ($P = 0.003$). There was no

difference in PSC duration before dysplasia or CCA diagnosis. Thirty (67%) patients had IBD. Twenty-two of these 30 patients had ulcerative colitis, seven patients had Crohn's disease, and one patient had indeterminate colitis. The presence of IBD was not significantly different between groups.

Laboratory values

ALP, bilirubin, AST and ALT values were not significantly different between the three groups at the latest ERC before LT or detection of CCA. CA19-9 was higher in CCA patients ($P = 0.002$). CEA values did not significantly differ between groups ($P = 0.081$).

ERC findings

There were no significant differences in ERC scores between patient groups (Table 2). Intrahepatic disease without extrahepatic changes was observed in ERC in only two patients, and both of these patients exhibited LGD. Only mild extrahepatic ERC changes (modified Amsterdam ERC score 1-2) were observed in 11 patients, and five of these patients exhibited CCA. Abundant extrahepatic ERC changes (score ≥ 3 , corresponding to dominant stricture) were observed in 32 patients. Fourteen (14/32) of these patients were diagnosed with CCA.

Brush cytology

All patients underwent ERC with BC (1-9 BC/patient, median 3 BCs). The number of available BCs did not significantly differ between groups (Table 3). Suspicious BC was observed in 11/16 patients with LGD, 8/10 patients with HGD and 4/4 patients with CCA in liver transplanted patients. Eleven of 15 patients without LT had suspicious or malignant BC (malignant 2 patients, suspicious 9 patients). Suspicious or malignant BC was observed in 34/45 patients (76%), with no difference between the neoplastic groups.

Ploidy analyses

Ploidy analysis of a brush sample was available for all patients (1-7/patient, median 3 DNA flow cytometry analyses). Aneuploidy was observed in 18/45 patients (40%), and there were no significant differences between groups. DNA indices were between 1 and 2.41 and did not significantly vary between the three groups. The median DNA index in HGD was 1.2.

Symptoms

Eighty percent of patients were symptomatic, with no difference between groups (Table 2). However, CCA patients were more often icteric than patients with LGD ($P = 0.014$).

Explanted livers

The median weight of explanted livers was 1399 g (range, 768-2195 g, IQR 1244-1526 g). The median

Table 2 Demographics, laboratory results and endoscopic retrograde cholangiography findings in patients with primary sclerosing cholangitis and low-grade dysplasia, high-grade dysplasia and cholangiocarcinoma

	LGD	HGD	CCA	<i>P</i> value	<i>P</i> value (LGD vs HGD)	<i>P</i> value (HGD vs CCA)	<i>P</i> value (LGD vs CCA)
<i>n</i>	16	10	19
Male/female	10/6	8/2	14/5	0.701	.	.	.
Age, yr	32 (26-48)	48 (35-59)	55 (37-60)	0.003	0.056	0.896	0.004
PSC duration, yr	4.1 (2.2-8.3)	4.4 (2.3-7.9)	4.0 (0.3-17.1)	0.948	.	.	.
Symptoms, <i>n</i>	12	8	16	0.890	.	.	.
Icterus, <i>n</i>	2	4	12	0.014	0.483	1.000	0.024
Pruritus, <i>n</i>	9	3	8	1.000	.	.	.
Cholangitis/fever, <i>n</i>	4	2	8	0.415	.	.	.
Fatigue, <i>n</i>	7	3	4	1.000	.	.	.
Pain, <i>n</i>	5	3	4	0.863	.	.	.
MELD score	7.6 (7.4-11.0)	7.6 (6.4-8.7)	7.7 (6.4-10.4)	0.883	.	.	.
IBD, <i>n</i>	10	5	15	0.307	.	.	.
ALP, U/L	175 (90-324)	89 (69-176)	237 (158-396)	0.214	.	.	.
AST, U/L	45 (32-118)	51 (34-64)	59 (38-94)	0.618	.	.	.
ALT, U/L	61 (31-113)	56 (30-102)	55 (46-112)	0.717	.	.	.
Bilirubin, μ mol/L	23 (13-32)	11 (8-22)	25 (15-47)	0.354	.	.	.
CA19-9, KU/L	10 (3-22)	10 (5-16)	120 (14-415)	0.002	1.000	0.006	0.006
CEA, μ g/L	1.3 (1.0-2.7)	2.4 (1.4-2.9)	2.1 (1.4-5.8)	0.081	.	.	.
mERC score							
Intrahepatic (IQR)	6 (4-6)	6 (5-7)	6 (6-6)	0.440	.	.	.
Extrahepatic (IQR)	3 (2-6)	4 (2-6)	4 (2-5)	0.613	.	.	.
Total score (IQR)	9 (7-11)	11 (8-12)	9 (8-11)	0.523	.	.	.

Values are presented as median (IQR) unless otherwise indicated. Jonckheere-Terpstra test was used to analyse differences of continuous variables, and Fisher's exact test was used for dichotomous variables. Significance values were adjusted using the Bonferroni correction for multiple tests. CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile rate; LGD: Low-grade dysplasia; mERC score: Modified Amsterdam ERC score; MELD score: Model for end-stage liver disease score; IBD: Inflammatory bowel disease; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PSC: Primary sclerosing cholangitis; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen.

Table 3 Brush cytology and DNA flow cytometry in patients with low-grade dysplasia, high-grade dysplasia or cholangiocarcinoma

	LGD	HGD	CCA	<i>P</i> value
Patients, <i>n</i>	16	10	19	
Liver transplanted, <i>n</i>	16	10	4	
BCs, <i>n</i> (IQR)	4.0 (2.3-5.0)	4.5 (1.8-5.3)	2.5 (1.8-5.3)	0.219
BC suspicious/malignant	11	8	15	0.746
Flow cytometry, <i>n</i> (IQR)	3.0 (2.3-4.0)	3.0 (1.8-5.0)	2.0 (1.0-4.3)	0.195
DNA-index (highest, IQR)	1.0 (1.0-1.2)	1.2 (1.0-1.8)	1.0 (1.0-1.2)	0.745
Aneuploid	6	5	7	0.795

Numbers are median (IQR) or *n*. *P* values: Jonckheere-Terpstra test for continuous variables, and Fisher's exact test for dichotomous variables. BC: Brush cytology; CCA: Cholangiocarcinoma; HGD: High-grade dysplasia; IQR: Interquartile rate; LGD: Low-grade dysplasia.

fibrosis stage was 3 (range 1-4^[8]), with no difference between the groups. Only mild fibrosis (stage 1-2) was observed in 3/16 of the patients with LGD, and 4/14 patients with HGD or CCA. The remaining patients exhibited a fibrosis stage of 3-4. Histologically typical PSC morphology was observed in all liver samples, including onion-skin fibrosis around the bile ducts and cholangitis. No dysplasia in peripheral bile ducts was observed. All patients with HGD also exhibited LGD of the bile ducts. All CCA patients also exhibited HGD of the bile ducts. Hepatocellular carcinoma was observed in two patients, who also exhibited LGD.

Cholangiocarcinoma

Nineteen patients exhibited CCA. Four CCAs were diagnosed in explanted livers after LT. Two CCAs were diagnosed at laparotomy during a planned LT. Two CCAs were detected at autopsy. A histological sample from the main tumour or metastasis was obtained during surgery or core-needle biopsy in eight patients. Two CCAs were verified with imaging and (malignant) BC, and one CCA was verified using imaging only. CCAs were diagnosed a median of 4.0 years after PSC diagnosis (range, 0-19 years). Six CCAs were detected less than one year after PSC diagnosis, two of these CCAs were detected at a diagnostic ERC. The localisation of CCA was perihilar or extrahepatic in 14 cases and intrahepatic in four cases. The origin of the tumour could not be determined in one patient with peritoneal carcinosis. The median time from the latest BC until CCA diagnosis was 53 d. BC was malignant in two CCA patients, and it remained suspicious in 13 patients. BCs were benign in four CCA patients. BC was suspicious in two of the patients with intrahepatic CCA and benign in two of these patients.

DISCUSSION

Forty-two patients with histologically confirmed biliary neoplasia were found during ERC surveillance: 16 patients with LGD, 10 patients with HGD and 16 patients with CCA. An additional 3 CCAs were

detected without histological confirmation. Patients with LGD were younger. AST, ALT or CEA values did not differ between the groups. CA19-9 was significantly elevated only in patients with CCA. No difference in PSC duration, ERC changes, suspicious BC, ploidy analysis, presence of IBD or histological fibrosis was found between the three groups. It was impossible to differentiate LGD from HGD based on liver enzymes, tumour markers, or BC. Evaluations for LT should already be performed when BC is repeatedly suspicious.

The number of cytological samples suspicious for neoplasia was equal in all three neoplastic groups. The sensitivity of BC to detect biliary neoplasia was quite low (43%) in earlier studies^[9]. The sensitivity to detect CCA with BC (neoplasia suspicion or malignant BC) in our previous study was up to 71% in a large, unselected patient population^[5]. This high accuracy was thought to be a result of the sampling method^[4] and the protocol used in our hospital. Two experienced gastroenterologists perform the ERCs, and the cellular yield is generally very high. A dedicated team of cytopathologists also perform the cytological interpretation. However, biliary neoplasia is not always found in the explanted liver, even with suspicious BC. Twenty-one patients exhibited benign biliary histology in the explanted liver in our earlier study, and four of these patients had suspicious BC^[5]. It would be clinically relevant to differentiate the HGD group from the other two neoplastic groups before LT because the timing of an early LT would likely exhibit the most benefit. However, we report that cytology alone did not separate three groups with neoplastic biliary changes. Neoplastic biliary changes were also not detected in liver biopsy because biliary dysplasia was not observed in the peripheral bile ducts. However, HGD may be diagnosed with BC in select cases^[10].

We also used DNA flow cytometry in addition to BC in selected cases (*e.g.*, advanced ERC changes or previously suspicious BC). Based on earlier studies^[7,11], aneuploidy was not more commonly observed in CCAs. This result may reflect the fact that cancer cells in CCA may not be reachable for brushing because of their growth pattern or desmoplasia. The median DNA index was higher in patients with HGD, but this difference was likely the result of the low number of cases, and statistical significance was not observed. The demand for more cases to reach significance is also based on the fact that only a portion of the dysplasias, or even CCAs, presented with aneuploid DNA. Better markers for HGD than aneuploidy are needed. The combination of genetic abnormalities with BC may help distinguish benign and malignant bile duct strictures^[12,13]. However, there are no specific markers to detect HGD without histology.

CA19-9 levels often rise with CCA, but it is not specific for CCA^[14,15]. CA19-9 may be markedly elevated in up to 32% of PSC patients in the absence of CCA^[15]. CA19-9 was elevated mostly in CCA patients in the

present study, which supports the use of CA19-9 as a tumour marker for CCA. However, it is not suitable for biliary dysplasia screening. Several surveillance algorithms of PSC recommend the use of CA19-9^[2,16,17]. Elevated CEA values were also commonly observed in CCA patients but only immediately prior to the CCA diagnosis (data not shown). These findings suggest that CA19-9 and CEA are late markers, and they cannot be used for the detection of premalignant lesions. The elevation of CA19-9 generally indicates that the dysplasia surveillance programme has actually failed to find cases with dysplasia. CA19-9 is not expressed in 5%-10% of the population who have the Lewis a- b-genotype^[18], and the fucosyltransferase genotype influences CEA levels^[19].

ALP is generally elevated at PSC diagnosis, and normalisation of ALP is associated with a better prognosis^[20-24]. ALP levels are not helpful in differentiating HGD from LGD, which was observed in this study. CCA patients were not younger than other patients, and there was up to 19 years of time between PSC and CCA diagnosis. One-third (6/19) of CCAs were detected within one year of PSC diagnosis in the present study. Ten of the 19 CCA patients in this study were diagnosed with PSC prior to the beginning of this study period (2008). Therefore, the effect of this surveillance programme could not be evaluated for the entire PSC population. Patients with LGD were significantly younger than patients with CCA or HGD. Retrospectively, some of these patients could have waited for the LT if there were no symptoms, but this study demonstrated that it is generally impossible to diagnose HGD prior to LT.

ERC findings were similar in all patient groups, which is likely due to several end-stage PSCs with cirrhosis in the LGD group. ERC with BC is routinely performed for the screening and detecting of biliary dysplasia before LT in our hospital, even LT due to end-stage cirrhosis or symptoms. Our results demonstrate that the ERC score is not helpful for the differentiation of these three groups. Our strategy significantly differs from the practice in many US transplantation centres, where pre-liver transplant screening for CCA in patients with PSC is routinely performed in only 30% of centres^[25].

LGD was observed in conjunction with HGD in all cases, and HGD of the bile ducts was observed in all four CCA patients who received LT. These CCAs were discovered after LT in the explanted liver. These findings support the dysplasia-CCA sequence as described by Lewis *et al.*^[3]. This theory of a dysplasia-cancer sequence, which was indirectly demonstrated in this study, justifies the dysplasia surveillance programme of PSC. However, further tools are needed to differentiate premalignant cases.

All HGDs and 14/19 CCAs were located in perihilar or extrahepatic bile ducts, which supports earlier studies that demonstrated that most CCAs were located in perihilar or distal bile ducts^[26]. CCA or dysplasia in

these locations is generally reachable using BC, and surveillance with BC may be justified. However, we observed 19 CCAs in these patients compared to 26 with premalignant lesions. One CCA was diagnosed in a patient who was dropped from the surveillance due to advanced age. Two CCAs were detected during the planned LT, and the LT was cancelled. Four CCAs were detected after LT, CCA was diagnosed in the explanted liver, and two of these patients had clean perioperative pathology samples. Two CCA patients were not under surveillance for years, but they returned with symptoms. The surveillance protocol was not optimal for all patients, and they entered the surveillance programme gradually. If HGD is considered the highest risk for CCA and patients diagnosed with CCA during the first year after PSC diagnosis are excluded, then at least 43% of CCAs were possibly prevented using early LT.

Early CCA is an indication for LT combined with chemo- and radiotherapy in select centres^[6]. Surveillance of biliary dysplasia using BC provides another option, with LT being performed at an earlier phase to avoid oncological treatment. The indications of LT in PSC patients in most centres include end-stage liver disease or symptomatic disease (*e.g.*, recurrent cholangitis). CCA is generally a contraindication for LT in our centre. LTs due to PSC have been performed since 1984 in our unit, and dysplasia in ERC has been considered a partial indication for LT in PSC patients since 2001^[27].

The limitation of this study is the small number of patients, which restrains the findings of this study, and some significant differences may be missed. Some patients who subsequently developed CCA did not follow the surveillance protocol until they became symptomatic. Our hospital is the only hospital in our country that performs organ transplantations. Therefore, no liver transplantations were missed during surveillance.

In conclusion, there is currently no reliable method to differentiate LGD from HGD prior to LT. CA19-9 rises with CCA but not in biliary dysplasia. Repeated suspicious BC, which suggests dysplasia and a high risk for future CCA, may be an indication for LT.

COMMENTS

Background

Primary sclerosing cholangitis (PSC) patients exhibit an elevated risk for cholangiocarcinoma (CCA), with a lifetime risk of 5%-10%. Biliary dysplasia is a precursor of CCA, and liver transplantation (LT) may be considered to prevent progression to CCA in cases with biliary dysplasia. A more common indication for LT is end-stage liver disease or disease symptoms. CCA develops via a low-grade dysplasia (LGD) to high-grade dysplasia (HGD) to CCA sequence. PSC patients in our centre undergo regular surveillance with endoscopic retrograde cholangiography (ERC) and brush cytology (BC) to detect biliary dysplasia, and patients with repeated suspicious findings are referred for evaluation of LT. The diagnosis of HGD prior to consideration for LT would be beneficial because of the high risk of progression to CCA. This study compared ERC, BC, ploidy analysis, and laboratory values between patients diagnosed with CCA, HGD or LGD.

Research frontiers

Biliary dysplasia is an established indication for LT in few transplantation centres. BC is used to detect biliary neoplasia, but HGD may be verified from brushing only in selected cases.

Innovations and breakthroughs

This study found that differentiating HGD and LGD prior to histological verification in the explanted liver is not practically possible. BC, laboratory values and ERC scores did not differ between patients with LGD, HGD and CCA, with the exception of CA19-9, which was elevated in CCA patients.

Applications

This study suggests that the patient should be referred for evaluation of LT when biliary neoplasia is repeatedly suspected in BC of a PSC patient to prevent progression to CCA.

Terminology

ERC - an endoscopic procedure that enables an evaluation of bile ducts by injecting contrast medium to the bile ducts for radiographic visualisation.

Peer-review

The paper is novel, and an interesting study evaluating clinical and laboratory characteristics of PSC patients in order to identify subjects with biliary dysplasia that could benefit from early liver transplantation. The figures are good and eye-catching.

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Retrospective Study

Management of gastric mucosa-associated lymphoid tissue lymphoma in patients with extra copies of the *MALT1* gene

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Abstract

AIM

To identify the clinical features of gastric mucosa-associated lymphoid tissue (MALT) lymphoma with extra copies of *MALT1*.

METHODS

This is a multi-centered, retrospective study. We reviewed 146 patients with MALT lymphoma in the stomach who underwent fluorescence *in situ* hybridization analysis for t(11;18) translocation. Patients were subdivided into patients without t(11;18) translocation or extra copies of *MALT1* (Group A, *n* = 88), patients with t(11;18) translocation (Group B, *n* = 27), and patients with extra copies of *MALT1* (Group C, *n* = 31). The clinical background, treatment, and outcomes of each group were investigated.

RESULTS

Groups A and C showed slight female predominance, whereas Group B showed slight male predominance. Mean ages and clinical stages at lymphoma diagnosis were not different between groups. Complete response was obtained in 61 patients in Group A (69.3%), 22 in Group B (81.5%), and 21 in Group C (67.7%). *Helicobacter pylori* (*H. pylori*) eradication alone resulted in complete remission in 44 patients in Group A and 13 in Group C. In Group B, 14 patients underwent radiotherapy alone, which resulted in lymphoma disappearance. Although the difference was not statistically significant, event-free survival in Group C tended to be inferior to that in Group A (*P* = 0.10).

CONCLUSION

Patients with t(11;18) translocation should be treated differently from others. Patients with extra copies of *MALT1* could be initially treated with *H. pylori* eradication, similar to patients without t(11;18) translocation or extra copies of *MALT1*.

Key words: Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue; Gastric neoplasms; Esophagogastroduodenoscopy; t(11;18) translocation; Trisomy 18

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Core tip: We subdivided and retrospectively reviewed 146 patients with gastric MALT lymphoma into patients without t(11;18) translocation or extra copies of *MALT1* (Group A, *n* = 88, 60.3%), patients with t(11;18)

translocation (Group B, *n* = 27, 18.5%), and patients with extra copies of *MALT1* (Group C, *n* = 31, 21.2%). Groups A and C exhibited similar clinical characteristics. *Helicobacter pylori* eradication alone resulted in complete remission in approximately half the patients in Group A and Group C. Consequently, patients with extra copies of *MALT1* could be treated similar to patients without t(11;18) translocation or extra copies of *MALT1*.

Iwamuro M, Takenaka R, Nakagawa M, Moritou Y, Saito S, Hori S, Inaba T, Kawai Y, Toyokawa T, Tanaka T, Yoshino T, Okada H. Management of gastric mucosa-associated lymphoid tissue lymphoma in patients with extra copies of the *MALT1* gene. *World J Gastroenterol* 2017; 23(33): 6155-6163 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6155.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6155>

INTRODUCTION

Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is one of the non-Hodgkin lymphomas, originating in tissues or organs outside of the lymph nodes. Typically, this neoplasm involves the gastrointestinal tract, thyroid, ocular adnexa, lungs, salivary glands, liver, and skin^[1,2]. Of these organs, the stomach is the most frequently identified primary site. Approximately two-thirds of patients with gastric MALT lymphoma have chronic *Helicobacter pylori* (*H. pylori*) infection in the stomach, which is believed to be the causative organism. Clinically, eradication of *H. pylori* is the first-line management of choice, resulting in regression of lymphoma in 75% to 80% of patients^[3]. Meanwhile, cases with chromosomal abnormalities of t(11;18)(q21;q21)/*API2-MALT1* translocation have been shown to be resistant to *H. pylori* eradication. Consequently, evaluation of t(11;18)(q21;q21) translocation at the initial workup is valuable to forecast the response to antibiotic therapy against *H. pylori*^[4].

Previously, we reported a case of gastric MALT lymphoma with trisomy 18^[5]. In that case, although there were no fusion genes of *API2-MALT1*, trisomy 18 was identified as extra copies of *MALT1*, using a fluorescence *in situ* hybridization (FISH) analysis for t(11;18)(q21;q21)/*API2-MALT1* translocation, since *MALT1* is located on chromosome 18q21. The patient required radiation therapy to treat the gastric lymphoma lesions, because the gastric lesions only partially improved after eradication therapy for *H. pylori*. Though radiation therapy resulted in complete remission, MALT lymphoma recurred in the stomach 16 mo later. Previous studies investigating chromosome aneuploidy in MALT lymphomas also reported that trisomy 18 might be indicative of progression or relapse in patients with gastric MALT

lymphoma^[6-8]. Based on our experience and previous reports, we hypothesized that gastric MALT lymphoma with extra copies of the *MALT1* gene may be resistant to *H. pylori* eradication and may show more frequent progression or relapse than those without chromosomal aberrations. However, no studies have described the initial treatment and response in such patients. The purpose of this study is to reveal the clinical characteristics and outcomes of gastric MALT lymphoma in patients with t(11;18)(q21;q21)/*API2-MALT1* translocation or extra copies of *MALT1*, in order to determine initial treatment strategies for cases presenting with chromosomal aneuploidy.

MATERIALS AND METHODS

Letters of inquiry regarding patients with gastric MALT lymphoma were sent from the Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, to ten collaborating institutions. The inclusion criteria were: (1) patients with pathologically diagnosed MALT lymphoma in the stomach, and (2) patients who underwent fluorescence *in situ* hybridization analysis for t(11;18)(q21;q21)/*API2-MALT1* translocation on biopsy samples from the gastric MALT lymphoma lesions. FISH analysis was performed for all patients on fresh biopsy samples using dual-color, dual-fusion translocation probes for *API2/MALT1*. MALT lymphoma with a large-cell component was excluded from this study. Finally, a total of 146 patients diagnosed with gastric MALT lymphoma between October 1997 and November 2015 were identified. Thus, these patients were retrospectively registered in this study.

According to the FISH result for t(11;18) translocation, patients were subdivided into three groups as follows: (1) Group A: patients without t(11;18)(q21;q21)/*API2-MALT1* translocation or extra copies of *MALT1* (Figure 1A), (2) Group B: patients with t(11;18) translocation (Figure 1B), and (3) Group C: patients with extra copies of *MALT1* (Figure 1C). To determine the patient characteristics of gastric MALT lymphoma with or without chromosomal aberrations, we retrospectively examined the gender, age at diagnosis, endoscopic features, clinical stages according to Lugano classification^[9], *H. pylori* infection status, treatments, response to treatments, and outcomes. *H. pylori* infection status was examined by urea breath tests, rapid urease tests, microscopic observations or culture tests on endoscopically biopsied specimens, stool antigen tests, serum or urine antibody tests, or a combination of these methods. Success of *H. pylori* eradication was confirmed by urea breath tests, rapid urease tests, microscopic observations or culture tests on endoscopically biopsied specimens, or stool antigen tests. The follow-up period was defined as the period from the lymphoma diagnosis to death from any cause or to the patient's last hospital visit.

Macroscopic features of gastric lymphoma lesions observed during esophagogastroduodenoscopy were classified into the following six subtypes^[10]: (1) erosions/ulcers; (2) early gastric cancer-like lesion that formed a slightly depressed area; (3) whitish mucosa; (4) cobblestone appearance; (5) submucosal tumor-like lesion; and (6) mixed (a combination of these five subtypes). All cases were reviewed and their subtypes were classified by board certified endoscopists.

Event-free survival was measured from diagnosis until documented progression/relapse, death from primary disease, or commencement of the second treatment for any reason. For the comparisons of the two groups, statistical analyses including *t*-tests, χ^2 tests, and *F*-tests were performed using JMP 8.0.1 software (SAS Institute, Cary, NC, United States). Cumulative event-free probabilities were calculated by Kaplan-Meier analysis and log-rank tests were performed using the JMP 8.0.1 software. *P* < 0.05 was considered to indicate a statistically significant difference. The present study was approved by the Ethical Committee of the Okayama University Hospital and adhered to the Declaration of Helsinki.

RESULTS

In this study, clinical data of 146 patients (76 women and 70 men) were collected from nine institutions (Table 1). FISH analysis for t(11;18)(q21;q21)/*API2-MALT1* revealed that t(11;18) translocation and extra copies of *MALT1* were not identified in 88 patients (Figure 1A). Fusion genes of *API2-MALT1* indicating t(11;18)(q21;q21)/*API2-MALT1* translocation were detected in 27 patients (10 women and 17 men) (Figure 1B). Extra copies of *MALT1* were found in 31 patients; the ratio of *MALT1* signals to *API2* signals was 3:2 in 28 patients, suggesting trisomy 18 (Figure 1C), and 4:2 in one patient, suggesting tetrasomy 18, (Figure 1D). The remaining two patients had a combination of lymphoma cells with either trisomy 18 or tetrasomy 18. In one patient with extra copies of *MALT1*, several lymphoma cells had six *MALT1* signals with four *API2* signals, which is considered as trisomy 18 with tetraploidy (Figure 1E).

Patients without t(11;18) translocation or extra copies of *MALT1* (Group A) showed slight female predominance (48 women and 40 men), while patients with t(11;18)(q21;q21) translocation (Group B) showed slight male predominance (10 women and 17 men). However, there was no statistically significant difference between Groups A and B regarding sex ratio (*P* = 0.11). Patients with extra copies of *MALT1* (Group C) showed slight female predominance (18 females and 13 males) compared to Group A (*P* = 0.73). The mean ages at lymphoma diagnosis was 65.9 ± 12.3 years in Group A, 64.2 ± 11.2 years in Group B, and 65.0 ± 14.9 years in Group C and was not different between groups. Lymphoma lesions were localized in the stomach without lymph node or other organ

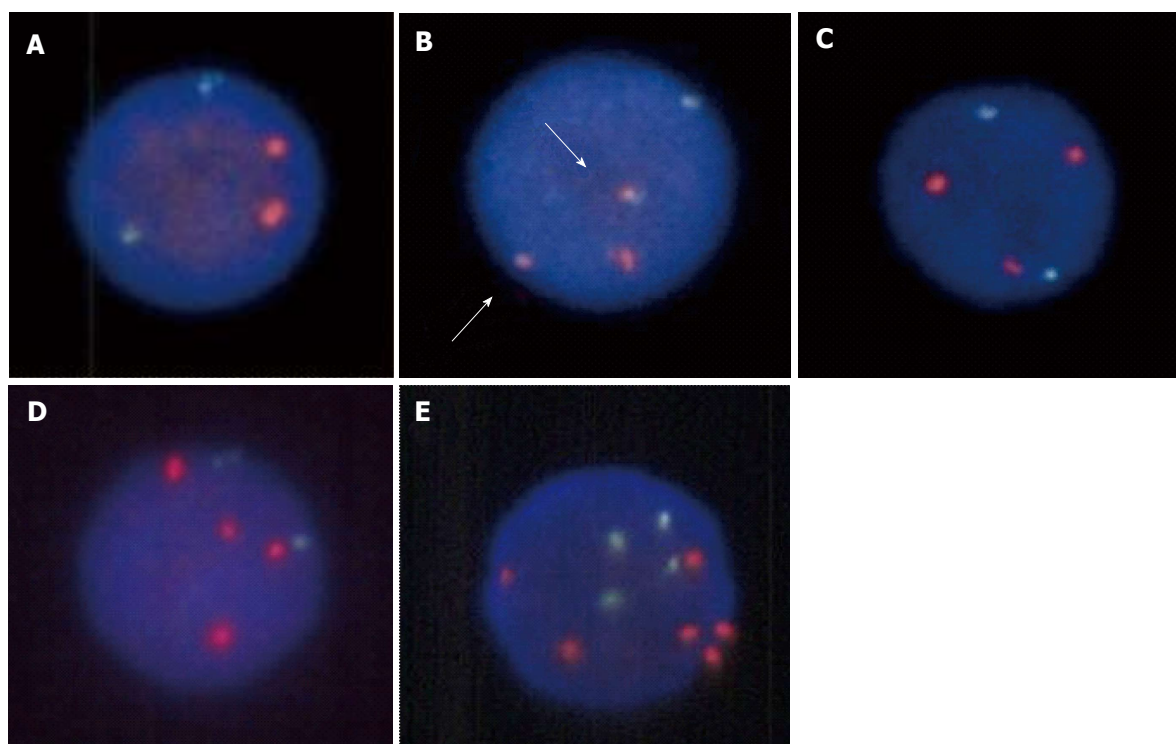


Figure 1 FISH images of the lymphoma cells. In the FISH analysis for t(11;18)(q21; q21) translocation, *API2* is visualized as a green signal and *MALT1* as a red signal. A: Two green signals and two red signals are seen in cases without t(11;18) translocation or extra copies of *MALT1*. B: Fusion genes of *API2-MALT1* are detected as yellow signals, indicating t(11;18) translocation (B, arrows). C: Although no fusion genes are visible, an extra copy of *MALT1* is noted, indicating trisomy 18. D: Two extra copies of *MALT1* are visible, indicating tetrasomy 18. E: In one patient, several lymphoma cells have six *MALT1* signals with four *API2* signals, which is considered as trisomy 18 with tetraploidy.

Table 1 Clinical backgrounds of the study subjects *n* (%)

	Total	Group A: No chromosome aberration ¹	Group B: t(11;18) positive	Group C: Extra copies of <i>MALT1</i>	Group A vs B, <i>p</i> value	Group A vs C, <i>p</i> value
<i>n</i>	146	88	27	31		
Sex						
Male	70 (47.9)	40 (45.5)	17 (63.0)	13 (41.9)	0.11	0.73
Female	76 (52.1)	48 (54.5)	10 (37.0)	18 (58.1)		
Age (mean ± SD, yr)	65.4 ± 12.6	65.9 ± 12.3	64.2 ± 11.2	65.0 ± 14.9	0.53	0.75
Stage (Lugano system)						
I	131	81	24	26	0.92 ²	0.29 ²
II 1	3	1	1	1		
II 2	1	1	0	0		
IV	11	5	2	4		
<i>H. pylori</i>						
Positive	89 (61.0)	66 (75.0)	2 (7.4)	21 (67.7)	< 0.01	0.439
Negative	57 (39.0)	22 (25.0)	25 (92.6)	10 (32.3)		

¹Patients without t(11;18) translocation or extra copies of *MALT1*; ²stage I and II 1 vs II 2 and IV.

involvement in most of the patients (131/146, 89.7%), thus they were classified as stage I according to the Lugano system of staging^[10]. *H. pylori* infection status was examined in all patients. In Group A, 75.0% of the patients were positive for *H. pylori*. Group C had similar positivity for *H. pylori* (67.7%). In contrast, only 7.4% of the patients in Group B had *H. pylori* infection ($P < 0.01$, vs Group A). Endoscopic features are summarized in Table 2. Overall, gross findings of the gastric lymphoma lesions were as follows: erosions/ulcers in 44 patients (30.1%), whitish mucosa

in 42 patients (28.8%), submucosal tumor-like lesions in 29 patients (19.9%), cobblestone appearance in 17 patients (11.6%), early gastric cancer-like lesions in 10 patients (6.8%), and mixed lesions in four patients (2.7%). The ratio of each macroscopic morphology was similar between Groups A and C. Moreover, no patients in Group B presented with submucosal tumor-like lesions.

Treatments and outcomes are shown in Table 3. Follow-up periods were 3.9 ± 3.1 years in Group A, 5.1 ± 4.5 years in Group B, and 2.9 ± 2.2 years in

Table 2 Endoscopic features of the gastric lesions *n* (%)

	Total	Group A: No chromosome aberration ¹	Group B: t(11;18) positive	Group C: Extra copies of <i>MALT1</i>
Macroscopic feature				
Erosions/ulcers	44 (30.1)	23 (26.1)	9 (33.3)	12 (38.7)
Early gastric cancer-like	10 (6.8)	7 (8.0)	2 (7.4)	1 (3.2)
Whitish mucosa	42 (28.8)	25 (28.4)	12 (44.4)	5 (16.1)
Cobblestone appearance	17 (11.6)	12 (13.6)	3 (11.1)	2 (6.5)
Submucosal tumor	29 (19.9)	19 (21.6)	0	10 (32.3)
Mixed	4 (2.7)	2 (2.3)	1 (3.7)	1 (3.2)

¹Patients without t(11;18) translocation or extra copies of *MALT1*.**Table 3** Treatment regimens and outcomes of the study subjects

	Total	Group A: No chromosome aberration ¹	Group B: t(11;18) positive	Group C: Extra copies of <i>MALT1</i>
Treatment				
Eradication alone	85	62	3	20
RT alone	23	8	15	0
Chemotherapy alone	8	3	2	3
RT and chemotherapy	2	1	0	1
Eradication RT	17	6	5	6
Eradication and chemotherapy	3	2	0	1
Eradication and RT and chemotherapy	2	1	1	0
None	6	5	1	0
Outcome				
Live without disease	103	61	21	21
Live with disease	33	20	5	8
Live, unknown disease status	6	5	1	0
Dead by other cause	3	1	0	2
Dead by MALT lymphoma	1	1	0	0
Follow-up period (mean ± SD, yr)	3.9 ± 3.3	3.9 ± 3.1	5.1 ± 4.5	2.9 ± 2.2

¹Patients without t(11;18) translocation or extra copies of *MALT1*. RT: Radiotherapy; MALT: Mucosa-associated lymphoid tissue.

Group C. Only four patients died during the follow-up period: One patient in Group A died 2.4 years after lymphoma diagnosis because of concomitant advanced lung cancer, although complete response was obtained for gastric MALT lymphoma after *H. pylori* eradication. Two patients in Group C died of pneumonia 1.1 years and 2.0 years after lymphoma diagnosis, respectively. The other patient in Group A had stage IV lymphoma involving the stomach, ileum, colon, rectum, and lymphadenopathies of the neck, supraclavicular, subphrenic, and retroperitoneum. The patient underwent chemotherapy with cyclophosphamide, vincristine sulfate, and prednisone, followed by rituximab monotherapy and yttrium-90 ibritumomab tiuxetan monotherapy. However, the patient died of refractory lymphoma 1.4 years after

Table 4 Treatment regimens resulted in complete remission

	Total	Group A: No chromosome aberration ¹	Group B: t(11;18) positive	Group C: Extra copies of <i>MALT1</i>
<i>n</i>	103	61	21	21
Treatment				
Eradication alone	58	44	1	13
RT alone	20	6	14	0
Chemotherapy alone	4	1	1	2
RT and chemotherapy	2	1	0	1
Eradication and RT	13	5	4	4
Eradication and chemotherapy	3	2	0	1
Eradication, RT and chemotherapy	1	0	1	0
None	2	2	0	0

¹Patients without t(11;18) translocation or extra copies of *MALT1*. RT: Radiotherapy; MALT: Mucosa-associated lymphoid tissue.

the initial diagnosis due to intestinal perforation. Since pathological analysis was waived because of the patient's deteriorated condition, it was unknown whether histologic transformation occurred in the terminal stage. All patients, except for the four mortality cases, were alive at the patient's last visit to each institution. Five patients in Group A and one in Group B did not undergo esophagogastroduodenoscopy during the follow-up period, thus their lymphoma status, whether remitted or unchanged, was unknown. Complete response was obtained in 61 patients in Group A (69.3%), 22 in Group B (81.5%), and 21 in Group C (67.7%). On the other hand, lymphoma lesions were partially remitted or unchanged in 20 patients in Group A (22.7%), four in Group B (14.8%), and eight in Group C (25.8%).

With regard to treatment regimens, most of the patients without t(11;18) translocation or extra copies of *MALT1* (Group A) were treated with *H. pylori* eradication alone (*n* = 62, 70.5%). More than half of the patients with t(11;18)(q21;q21) translocation (Group B) were treated with radiotherapy alone (*n* = 15, 55.6%). Approximately two-thirds of the patients with extra copies of *MALT1* (Group C) were treated with *H. pylori* eradication alone (*n* = 20, 64.5%). Table 4 shows treatment regimens that resulted in complete remission of MALT lymphoma lesions. Complete remission of lymphoma was observed in

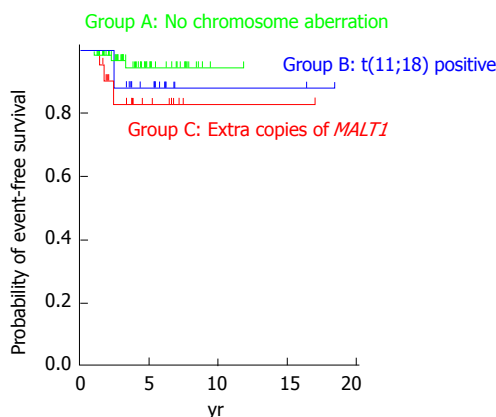


Figure 2 Cumulative event-free probabilities for the three groups. Although event-free survival of Group C patients appears to be inferior to that of the patients of the other two Groups, log-rank test revealed that the difference between Groups A and C was not statistically significant ($P = 0.10$).

44 (72.1%) and 13 (61.9%) patients in Groups A and C, respectively, by *H. pylori* eradication alone. In contrast, only one (4.5%) patient achieved complete remission after *H. pylori* eradication alone, whereas 14 (63.6%) patients achieved complete remission after radiotherapy alone in Group B. Figure 2 shows cumulative event-free probabilities between the three groups. Although event-free survival of Group C patients seems to be inferior than that of the Group A and Group B patients, log-rank test revealed that the difference between Groups A and C was not statistically significant ($P = 0.10$).

DISCUSSION

To our knowledge, the present report is the largest retrospective study investigating chromosome aberrations in gastric MALT lymphoma. Our review of 146 patients revealed that 31 patients (21.2%) had extra copies of *MALT1*. Prevalence of trisomy 18 in MALT lymphomas has been reported to range from 5% to 39%^[11-17]. Other chromosomal abnormalities involved in MALT lymphomas are trisomies 3, 7 and 12. The incidence varies between reports in the literature: trisomy 3 can be detected in 6% to 24%, trisomy 7 in 3% to 15%, and trisomy 12 in 3% to 38%^[17].

The clinical significance of these chromosomal numerical changes has been investigated in several studies^[6-8,18-20]. Tanimoto *et al.*^[19] investigated 34 patients with primary ocular adnexal MALT lymphoma and found that disease recurrence was documented in five cases, all of which had trisomy 18. Statistical analysis revealed that the time to recurrence was significantly shorter in patients with trisomy 18 than in those without trisomy 18 ($P = 0.05$). We also reported a patient with gastric MALT lymphoma with trisomy 18 in whom only partial improvement was documented after eradication therapy for *H. pylori*,

and lymphoma recurred in the stomach 16 months after radiotherapy^[5]. On the other hand, Taji *et al.*^[17] retrospectively reviewed 13 patients with localized MALT lymphoma in the stomach, and reported that all patients with unresponsive or progressive disease had t(11;18) translocation or trisomy 3. Conversely, one patient with complete response had trisomy 18, and another patient with complete response had both trisomies 12 and 18. Based on these findings, the authors speculate that trisomy 3 is related to resistance to *H. pylori* eradication therapy and disease recurrence in gastric MALT lymphoma, whereas trisomies 12 and 18 are not. Nakamura and colleagues reviewed 90 cases of gastric MALT lymphoma and reported that extra copies of *MALT1* were found in 18 of 71 (25%) cases^[6]. Although overall survival was not associated with the presence of extra copies of *MALT1*, disease progression or relapse of lymphoma was more frequently observed in patients with extra copies of *MALT1*. Possible correlations between extra copies of *MALT1* and progression or relapse of lymphoma have been described in other studies as well^[7,8]. In the present study, although the difference was not statistically significant, log-rank test revealed similar tendencies (Figure 2). Consequently, patients with additional copies of *MALT1* may require more frequent clinical follow-up.

In the present study, the sex ratio of the patients with extra copies of *MALT1* (Group C) was similar to that of the patients without t(11;18) translocation or extra copies of *MALT1* (Group A), with the number of females being greater than males. On the contrary, although the difference was not statistically significant, patients with t(11;18) translocation (Group B) included more males than females. It was also noteworthy that Group C patients had similar prevalence of *H. pylori* infection to Group A. Other patient characteristics, such as the age at lymphoma diagnosis, clinical stages, and macroscopic morphology were not different between Groups A and C. The majority of Group A and Group C patients were treated with *H. pylori* eradication alone. Moreover, *H. pylori* eradication alone resulted in complete remission of lymphoma in 44 patients in Group A and 13 in Group C. Overall, the results observed in this study suggest that patients with trisomy and tetrasomy 18 require no distinct treatment from patients without aberrations. To our knowledge, the present study is the first to reveal clinical responsiveness to *H. pylori* eradication in gastric MALT lymphoma patients with extra copies of *MALT1*. In addition, in the present study, FISH analysis was performed by using fresh biopsy samples, whereas previous reports primarily involved analyses of archival pathologic specimens with the aim of clarifying the etiology of chromosomal aberrations. We believe that our results would be more readily interpreted in actual clinical settings for physicians responsible for gastric MALT lymphoma patient therapy.

The present study included 27 patients (18.5%)

with t(11;18)(q21;q21)/*API2-MALT1* translocation. t(11;18)(q21;q21)/*API2-MALT1* is the most frequent translocation among gastric, pulmonary, intestinal, and cutaneous MALT lymphomas, accounting for 13% to 35% of cases^[3,21,22]. Other chromosomal aberrations detected in MALT lymphomas include t(1;14)(p22;q32), t(14;18)(q32;q21), t(3;14)(q27;q32), and t(3;14)(p14.1;q32)^[3]. Several of such karyotypic alterations have been known to activate the nuclear pathway, which is considered to lead to lymphomagenesis. Thus, these translocation-positive MALT lymphomas arise independent of *H. pylori* infection and are unresponsive to antibiotic treatment^[3]. Moreover, these patients often show a late response and lymphoma relapse during follow-up^[18,23]. Regardless of the adverse clinical features, t(11;18) translocation is infrequently identified in transformed MALT lymphoma or diffuse large B-cell lymphoma, indicating that high-grade transformation is less likely to occur in translocation-positive MALT lymphomas than in those without chromosome aberration^[3,8,18,24]. In this context, polymerase chain reaction or FISH analysis for t(11;18) translocation is essential during the initial workup to determine the appropriate treatment strategy^[25]. Of additional clinical interest is that trisomy and tetrasomy 18 can be detected as extra copies of *MALT1* by FISH analysis, since *MALT1* is located on chromosome 18^[18,20].

In our study, all patients with t(11;18) translocation (Group B), except two, were negative for *H. pylori* infection. With regard to the macroscopic morphology of the gastric lesions, no patients in Group B presented with submucosal tumor-like lesions (Table 2). We speculate that this result reflects the lower proliferative potential of MALT lymphoma cells with t(11;18) translocation, compared to that of cells without t(11;18) translocation or extra copies of *MALT1*. Since almost no patients in Group B had *H. pylori* infection, most of the patients were treated with radiotherapy and/or chemotherapy. These clinical features were concordant with previously reported characteristics of MALT lymphoma with t(11;18) (q21;q21)/*API2-MALT1* translocation^[8,22-24].

There were several limitations associated with this study. First, the presence of extra copies of *MALT1* is often suggestive of partial or complete trisomy/tetrasomy 18; both are different. Trisomy/tetrasomy 18 should be determined only when extra copies are identified by FISH using centromere-specific probes for chromosome 18, or when gains of chromosome 18 are confirmed by array comparative genomic hybridization or by cytogenetic analysis (G-banding). However, these assays could not be performed because of the retrospective nature of this study. Second, not all chromosome alterations were investigated. As described above, trisomies 3, 7 and 12 and t(14;18) and t(3;14) translocations can be involved in MALT lymphomas^[26,27]. Therefore, Group A may have been heterogeneous and included

patients with chromosomal aberrations other than t(11;18) translocation and trisomy 18. Third, only patients who underwent FISH analysis for t(11;18) translocation were enrolled in this study. FISH analysis may be waived for patients in which *H. pylori* was eradicated immediately following the diagnosis of MALT lymphoma and lymphoma regression was achieved. Thus, selection bias is a possibility in this study. Fourth, because this is a retrospective, multi-centered study, treatment strategies of each patient may have varied according to the attending physician's preference and the availability of chemotherapy and/or radiotherapy at each institution. Fifth, methods to examine *H. pylori* infection status varied among the different institutions participating in this study. Prospective studies utilizing uniform protocols are required to definitively compare treatment responses and prognosis in each group.

In conclusion, we reviewed 146 patients, including 31 (21.2%) with extra copies of *MALT1* and 27 (18.5%) with t(11;18)(q21;q21)/*API2-MALT1* translocation. Patients with t(11;18) translocation should be treated differently from others. However, patients with extra copies of *MALT1* have similar clinical characteristics to cases without t(11;18) translocation or extra copies of *MALT1*. We propose that these patients can be initially managed in the same way as cases without chromosomal aberrations. However, since progression or relapse tended to be more frequently seen in patients with extra copies of *MALT1*, these patients may require closer clinical follow-up.

COMMENTS

Background

Previous studies investigating chromosome aneuploidy in extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) reported that trisomy 18 might be indicative of progression or relapse in patients with gastric MALT lymphoma.

Research frontiers

No studies have described the initial treatment and response in patients with extra copies of *MALT1*.

Innovations and breakthroughs

Patients with extra copies of *MALT1* have similar clinical characteristics to cases without t(11;18) translocation or extra copies of *MALT1*. The present study is the first to reveal clinical responsiveness to *Helicobacter pylori* eradication in gastric MALT lymphoma patients with extra copies of *MALT1*.

Applications

Patients with extra copies of *MALT1* can be initially managed in the same way as cases without chromosomal aberrations.

Terminology

MALT lymphoma is one of the non-Hodgkin lymphomas, originating in tissues or organs outside of the lymph nodes. Typically, this neoplasm involves the gastrointestinal tract, thyroid, ocular adnexa, lungs, salivary glands, liver, and skin. Of these organs, the stomach is the most frequently identified primary site.

Peer-review

This is a well-designed multicenter study on the role of extra copies of *MALT1*

in gastric MALT lymphoma. The conclusion can be supported by the results. It can add something interest to current knowledge.

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Retrospective Study

Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy

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Abstract

AIM

To investigate whether patients with refractory epilepsy and healthy infants differ in gut microbiota (GM), and how ketogenic diet (KD) alters GM.

METHODS

A total of 14 epileptic and 30 healthy infants were recruited and seizure frequencies were recorded. Stool samples were collected for 16S rDNA sequencing using the Illumina Miseq platform. The composition of GM in each sample was analyzed with MOTHUR, and inter-group comparison was conducted by R software.

RESULTS

After being on KD treatment for a week, 64% of epileptic infants showed an obvious improvement, with a 50% decrease in seizure frequency. GM structure in epileptic

infants (P1 group) differed dramatically from that in healthy infants (Health group). Proteobacteria, which had accumulated significantly in the P1 group, decreased dramatically after KD treatment (P2 group). *Cronobacter* predominated in the P1 group and remained at a low level both in the Health and P2 groups. *Bacteroides* increased significantly in the P2 group, in which *Prevotella* and *Bifidobacterium* also grew in numbers and kept increasing.

CONCLUSION

GM pattern in healthy infants differed dramatically from that of the epileptic group. KD could significantly modify symptoms of epilepsy and reshape the GM of epileptic infants.

Key words: Ketogenic diet; *Cronobacter*; Seizures; Gut microbiota; Epilepsy

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Core tip: Many infants with epilepsy are refractory to current antiepileptic drugs, and ketogenic diet (KD) could help to moderate seizure frequency as an alternative treatment. A large number of reports have demonstrated that gut microbiota (GM) can affect children's neurodevelopment. Concurrently, GM could be dramatically affected by diet. KD could rapidly alter GM and alleviate seizure frequency in infants with refractory epilepsy. The GM structure of epileptic infants - comprising large numbers of pathogens, such as *Streptococcus* - differed from that of healthy controls. After KD therapy, GM of epileptic patients changed significantly, with fewer pathogens and more beneficial bacteria.

Xie G, Zhou Q, Qiu CZ, Dai WK, Wang HP, Li YH, Liao JX, Lu XG, Lin SF, Ye JH, Ma ZY, Wang WJ. Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. *World J Gastroenterol* 2017; 23(33): 6164-6171 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6164.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6164>

INTRODUCTION

Pediatric epilepsy is widespread, with complications including cognitive impairment, delayed neurodevelopment and loss of bodily control^[1,2]. Disequilibrium between excitation and depression of the central nervous system is acknowledged as the main factor in epilepsy incidence^[3]. Prior reports have identified increased inflammatory reactions and pro-inflammatory cytokines, such as interleukin (IL)-6, IL-17 and interferon, in the cerebrospinal fluid (CSF)^[4]. Anti-epileptic drugs (AEDs) and surgery are the main conventional treatments for infants with epilepsy^[5].

However, there are still 30% of epileptic infants who suffered from therapeutic futility and recurrent attacks.

A growing number of reports indicated that KD is a promising therapeutic alternative for infants with refractory epilepsy, as it has been shown to ameliorate their clinical symptoms, including the frequency of seizures^[6-10]. It remains unclear exactly how this occurs. Several reports implicated changed neurotransmitters after KD therapy, including γ -aminobutyric acid (GABA), monoamines and glutamate^[7,11]. Dahin *et al*^[12] and Freeman *et al*^[13] also identified increased ketone bodies (KBs) and decreased dopamine and serotonin^[12,13]. However, Sario-Jamardo *et al*^[14] found little change of neurotransmitters, pterins and amino acids in the CSF of KD responders as opposed to non-responders. These discrepant findings suggested a need for the further elucidation of the mechanisms of KD therapy.

Several studies showed that diet posed a significant effect on GM^[8,15]. A high-fat diet induced selective enrichment of bile-metabolizing microbiota, such as *Bacteroides*^[16], whilst high-fiber foods promoted the accumulation of plant-polysaccharide fermenting microbial organisms, including *Prevotella* and *Clostridium*^[16]. A number of reports implicated involvement of GM in enteric nervous system, blood-brain barrier and glial cell development, all of which were pivotal to behavioral control and cognitive progression^[17,18]. GM could produce neurotransmitters and gut hormones directly^[19] or indirectly by producing signaling molecules to regulate host cells^[20]. GM-derived short-chain fatty acids (SCFAs) could stimulate enterochromaffin cells to produce serotonin^[21]. Wikoff *et al*^[22] also documented decreased serotonin in peripheral serum in the absence of GM. Moreover, *Clostridium sporogenes* and *Ruminococcus gnavus* promoted decarboxylation of tryptophan to tryptamine, which modulated mood and appetite through amine-associated receptors^[23]. Based on the involvement of GM in the gut-brain axis, increasing reports demonstrated imbalanced GM in neurogenic diseases (NDs), including autism-spectrum disorder, Parkinson's disease, and depression^[24]. However, GM dysbiosis in childhood epilepsy remains unexplored.

Previous studies declared that short-term dietary intake could rapidly alter human GM^[8,15]. In this study, we performed a comparison between diseased infants (before and after KD treatment) and healthy controls, to explore if and how GM of infants with refractory epilepsy differed with that of age-matched healthy subjects. We also evaluated the therapeutic effect of KD on refractory epilepsy and the changes in GM after treatment. It is hoped that this research will help to bridge some gaps in the current understanding of refractory childhood epilepsy.

MATERIALS AND METHODS

Sample collection

We enrolled 14 pediatric patients with refractory

epilepsy (aged 1.95 ± 3.10 years, 11 male and 3 female) in Shenzhen Children's Hospital, according to the following inclusion criteria: Convulsion more than four times per week after treatment with ≥ 3 AEDs; no antibiotic exposure for at least 1 mo; no known genetic metabolic disorders or severe systemic illnesses; and successive KD therapy for at least 1 wk. KD was provided by Zeneca (Shenzhen, China), including Qitong ketogenic liquid milk (3.4 g protein, 8.0 g lipid and 0.6 g carbohydrate per 100 g milk), Qitong ketogenic cookies and Qitong ketogenic set-meal packages^[25].

Healthy subjects (aged up to 3 years, 15 male and 15 female) were also recruited based on the following criteria: No antibiotic exposure for at least 1 mo before this study, no disease symptoms for at least 1 mo following recruitment, and no history of seizures (Supplementary Table 1). Fisher's exact test was used to evaluate the effect of gender and age on GM composition.

DNA extraction, library construction and sequencing

The genomic DNA of microbiota was extracted from stool samples using the Power Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad) following the manufacture's protocol. The hypervariable V3-V4 region of the 16S rRNA gene was amplified using PCR kit (TransGenAP221-02, Peking), and DNA products were quantified by gel electrophoresis and Qubit (Thermo Fisher, Singapore). After library construction, the qualified libraries were sequenced using the Illumina MiSeq Sequencing platform (Illumina, San Diego).

Taxonomy classification and diversity detection

After filtration, overlapped paired reads were assembled as tags with FLASH (v1.2.11), and clustered to operational taxonomic units (OTUs) through USEARCH (v7.0.1090)^[26]. Representative OTUs were mapped against the Greengenes database (v201305)^[27] and classified with RDP classifier (v2.2)^[28]. The diversity of microbiota was calculated with MOTHUR (v1.31.2)^[29].

Principal component analysis and statistical analysis

PCA was performed with R software (v3.2.5). Wilcoxon rank-sum test was used to compare GM in diseased infants and healthy controls (Health group). Comparative analysis between the epileptic infants before (P1 group) and after treatment (P2 group) was conducted by Wilcoxon signed-rank test. Linear discriminant analysis Effect Size (LEfSe) analysis was used to identify microbial species which were apparently enriched in a specific group.

RESULTS

Data output and patients' characteristics

The average number of high-quality sequencing reads produced for each sample was 117196 (range, 31900

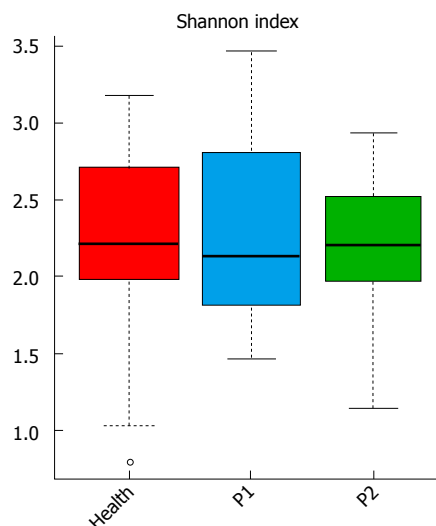


Figure 1 Gut microbial diversity of the three groups. Distribution of Shannon index (evenness) is shown. Red, blue, and green represent the Health, P1 and P2 groups, respectively. The gut microbiota (GM) of the healthy infants was more stable than that of the other two groups.

to 305190). The number of assembled tags averaged 22800, with a range from 12655 to 27337. Both gender and age had no significant effect on GM ($P = 0.069$ and 0.234 , respectively).

GM of healthy individuals differs dramatically with that of diseased infants

Shannon index analysis indicated higher GM diversity in healthy infants, in comparison with infants with refractory epilepsy (Figure 1, Supplementary Table 2). PCA of GM profile also identified that healthy infants could be clearly distinguished from patients (Figure 2, Supplementary Table 3). The phylum Firmicutes predominated in patients (45.82%) and was unchanged after KD therapy (47.00%) (Supplementary Table 4). Bacteroidetes accounted for 53.01% of GM in healthy infants, followed by Firmicutes (34.38%). After KD treatment, Bacteroidetes increased from 26.75% to 38.71%. Actinobacteria was enriched in healthy infants (8.49%) and occupied a lower percent in patients (2.38% before treatment and 2.92% after treatment). Proteobacteria was highly accumulated in infants with refractory epilepsy (24.34%) and decreased dramatically after KD therapy (10.77%). At the genus level, *Cronobacter* was dominant in the patients (23.30% vs 0.00% in the healthy group). By contrast, healthy subjects harbored more than twice *Bacteroides* (42.68%) than infants with refractory epilepsy (17.93%). *Prevotella* and *Bifidobacterium* also accumulated in the healthy group (7.25% and 7.84%, respectively) (Supplementary Table 5).

KD therapy ameliorates epilepsy and GM of patients started to improve

After a week of KD therapy, 3 (21%) patients were seizure-free and 6 (43%) had a 50% to 90% decrease of seizure frequency (Supplementary Table 1). The

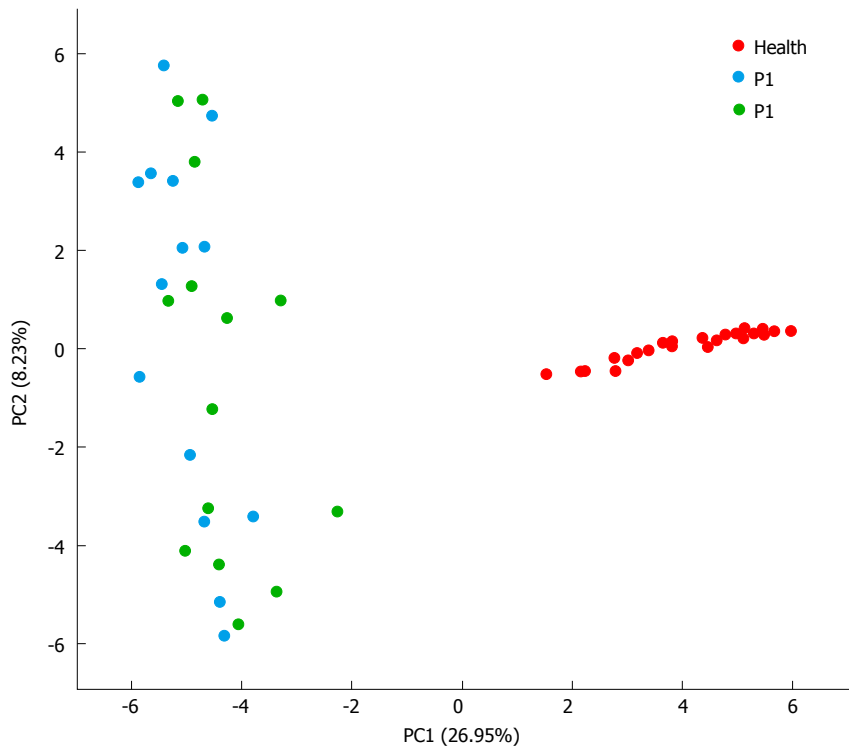


Figure 2 Principal component analysis. Each plot in the principal component analysis (PCA) graph stands for a sample. Red, blue and green colors represent the Health, P1 and P2 groups, respectively.

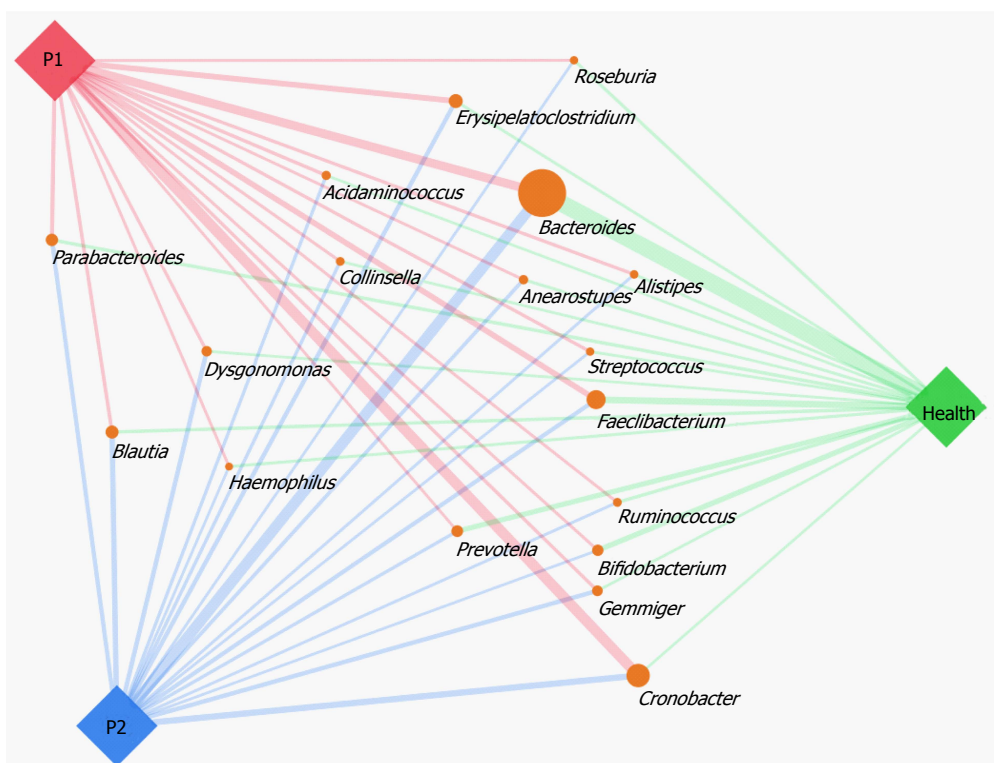


Figure 3 Gut microbiota structures in the Health, P1 and P2 groups at the genus level. SVG package (version 1.1) was used to produce the paragraph. The size of the circle representing each genus was determined by the relative abundance of the three groups, and the width of line linking the P1, P2 and Health groups indicates the relative abundance of each group.

remaining 5 (36%) infants experienced no significant improvement in seizure control (Supplementary Table 1). GM of the P2 group was more similar to that

of the Health group, by comparison with P1 group (Figures 3 and 4). After KD treatment, *Bacteroides* increased significantly, by 24.42%. *Prevotella* also

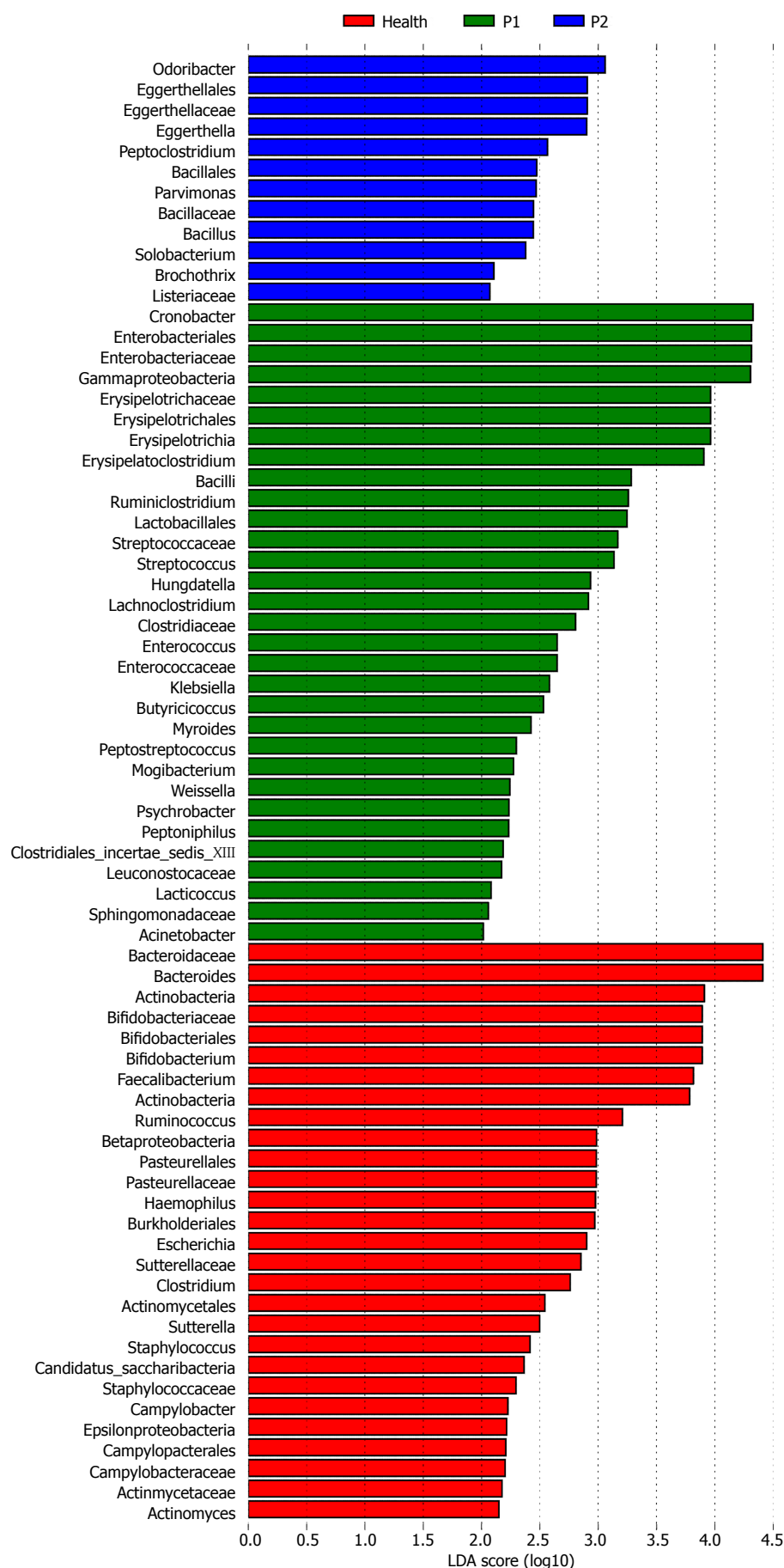


Figure 4 Significantly enriched gut microbiota components in the Health, P1 and P2 groups. LEfSe analysis was applied to detect the gut microbiota (GM) components in the three groups. Red, green, and blue represent the Health, P1 and P2 groups, respectively. The LDA score was set as ≤ 2 . The enrichment degree is proportional to the LDA score.

increased in the P1 group from 0.37% to 1.85% after KD treatment (Figure 3 and Supplementary Table 5). *Cronobacter* decreased sharply in after-treatment patients, from 23.3% to 10.44 % (Figures 3 and 4 and Supplementary Table 5). KD exposure also induced a decrease in *Erysipelatoclostridium* (by 8.67% in the P1 group and 4.89% in the P2 group); it represented just 0.64% in healthy infants (Figures 3 and 4 and Supplementary Table 5). *Streptococcus*, *Alistipes*, *Ruminiclostridium*, *Barnesiella* and *Enterococcus* also decreased after KD therapy (Figures 3 and 4 and Supplementary Table 5).

DISCUSSION

KD is increasingly used for the treatment of refractory epilepsy in childhood, but the mechanism remains unclear. Previous reports indicated that GM played an important role in the gut-brain axis^[24], and was affected significantly by intake of high-fat food^[16]. This study focused on differed GM structures between healthy and epileptic infants, as well as altered GM in patients after one week of KD treatment. The results pointed to an imbalanced GM in patients and a significant improvement after KD therapy.

Proteobacteria comprises a variety of notorious pathogens, such as *Escherichia*, *Salmonella* and *Vibrio*. It accounted for 24.34% in pediatric patients and decreased dramatically after KD treatment. Bacteroidetes was dominant in healthy infants and increased largely in after-treatment patients.

We identified accumulated *Bacteroides* in healthy subjects as well as in patients after treatment. *Bacteroides* was reported to digest and metabolize high-fat food and to regulate the secretion of IL-6 and IL-17 in dendritic cells (DCs), a process strongly associated with seizure severity of epileptic patients^[4,16]. However, patients-enriched *Cronobacter* decreased dramatically after KD therapy. Prior reports demonstrated that there were multiple virulence determinants of *Cronobacter*, including *Cronobacter* plasminogen activator and ferric ion transporter protein, which play a detrimental role in human health^[30-32]. *Prevotella* is a robust producer of SCFAs^[33], which could protect the intestinal mucosa and function as neurotransmitters. Previous reports also indicated that SCFAs mediated nervous impulse and mitigated Parkinson's disease^[33,34]. Similarly, we identified increased *Prevotella* in the Health and P2 group, when compared with the P1 group. Some other genera also offer clues to epilepsy recovery, such as *Erysipelatoclostridium*, *Blautia*, *Bifidobacterium* and *Streptococcus*. *Bifidobacterium* was well known to be beneficial to health^[35], and *Streptococcus*, a common pathogen, played a role especially in respiratory diseases^[36]. Although GM imbalance in diseased infants was identified and GM improved after KD treatment, more exploration was needed to elucidate the contribution of a healthy GM to epilepsy onset/recovery.

This study revealed that KD can mitigate the sym-

ptoms of epilepsy and correct an imbalanced GM in epileptic infants. However, further analysis is needed to unravel how GM may be involved in epilepsy onset/recovery.

There are some limitations that need to be clarified. First, 16S rDNA analysis identified microbes at the genus level, which makes it difficult to unravel different microbes at the species or function level. Second, it would be more useful to evaluate the efficacy of KD treatment and its effect on the GM if this could be done with a longer period of follow-up. Third, an animal model might be applied to demonstrate whether GM imbalance could induce epilepsy associated symptoms. Considering these limitations, we are planning to perform metagenomic analysis on GM of healthy and epileptic infants. This will provide more insights into distinct metabolic networks in imbalanced GM.

In conclusion, we found that GM of infants with refractory epilepsy differed dramatically from that of healthy infants. Epileptic patients harbored significantly enriched pathogens and decreased beneficial bacteria. Although this study provides new insight into the involvement of GM in pediatric refractory epilepsy, the gap between KD and epilepsy recovery is still huge. To uncover the mechanism and pathogens involved in refractory infantile epilepsy, further research should underscore functional gene networks in GM.

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COMMENTS

Background

Infants with refractory epilepsy could not be cured by several anti-epileptic drugs (AEDs) and ketogenic diet (KD) was increasingly used as an alternative therapy to refractory epilepsy. High-fat diet was reported to pose a significant impact on gut microbiota (GM), which could regulate neural systems.

Research frontiers

Previous reports demonstrated that GM could affect neural systems by secreting metabolites as neurotransmitters. In parallel, the gut-brain axis is a research hot spot in biomedicine, including the study of autism, Parkinson's disease, and depression.

Innovations and breakthroughs

This study showed that the GM pattern of diseased infants differs significantly from that of healthy controls. The decreased number of dominant pathogens and significantly increased number of beneficial bacteria after KD treatment offer new insight into KD therapy for epilepsy.

Applications

This study found several types of bacteria altered in the GM, suggesting that these bacteria could be monitored as biomarkers to provide an important reference for epilepsy treatment.

Terminology

GM, which consists of many kinds of bacteria including pathogens, commensals,

and probiotics, plays an important role in the human body.

Peer-review

The authors have performed important research in pediatric epilepsy. They discovered that the composition of the GM in healthy and diseased infants was significantly different, specifically in healthy infants as opposed to those with refractory epilepsy. Bacterial patterns were dramatically changed after KD therapy, and this was associated with a reduction in the frequency of seizures. These findings should enhance our knowledge of the relationship between epilepsy and GM and provide new insight into the clinical treatment of epilepsy.

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Observational Study

Definition of colorectal anastomotic leakage: A consensus survey among Dutch and Chinese colorectal surgeons

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Abstract

AIM

To determine the level of consensus on the definition of colorectal anastomotic leakage (CAL) among Dutch and Chinese colorectal surgeons.

METHODS

Dutch and Chinese colorectal surgeons were asked to partake in an online questionnaire. Consensus in the online questionnaire was defined as > 80% agreement between respondents on various statements regarding a general definition of CAL, and regarding clinical and radiological diagnosis of the complication.

RESULTS

Fifty-nine Dutch and 202 Chinese dedicated colorectal

surgeons participated in the online survey. Consensus was found on only one of the proposed elements of a general definition of CAL in both countries: 'extravasation of contrast medium after rectal enema on a CT scan'. Another two were found relevant according to Dutch surgeons: 'necrosis of the anastomosis found during reoperation', and 'a radiological collection treated with percutaneous drainage'. No consensus was found for all other proposed elements that may be included in a general definition.

CONCLUSION

There is no universally accepted definition of CAL in the Netherlands and China. Diagnosis of CAL based on clinical manifestations remains a point of discussion in both countries. Dutch surgeons are more likely to report 'subclinical' leaks as CAL, which partly explains the higher reported Dutch CAL rates.

Key words: Colorectal anastomotic leakage; Colorectal surgery; Definition; Complication

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Core tip: The present international online survey proves the inconsistent views as to what is considered colorectal anastomotic leakage among surgeons in the Netherlands and China, and shows large differences between the countries. This is in line with the current literature, since there is no uniformly accepted definition worldwide. We therefore propose to perform a systematic literature review to identify the available definitions. The final stage would be to perform a Delphi analysis within a representative panel of colorectal surgeons to develop a widely accepted definition of colorectal anastomotic leakage.

van Rooijen SJ, Jongen ACHM, Wu ZQ, Ji JF, Slooter GD, Roumen RMH, Bouvy ND. Definition of colorectal anastomotic leakage: A consensus survey among Dutch and Chinese colorectal surgeons. *World J Gastroenterol* 2017; 23(33): 6172-6180 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6172.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6172>

INTRODUCTION

Colorectal anastomotic leakage (CAL) remains gastrointestinal surgeons' most feared complication, despite important improvements in perioperative care and the development of novel surgical techniques. It is associated with high rates of morbidity and mortality^[1,2], poor quality of life^[3], and increased healthcare costs^[4,5]. Since CAL influences the direct postoperative course and has recently been proven to impact oncological outcome as well^[6-8], it is frequently used as an outcome measure in clinical studies. However, the CAL rates vary considerably in

the international literature from 1.5% to 23%^[9,10]. Large variations in leakage rates have been reported between studies published by Western and Asian research groups, in which the reported incidence of CAL in Asian publications is substantially lower^[5,11-14]. Such differences can be partly explained by the variations of operation technique, tumor location, and patient characteristics^[15,16]. However, little attention has been paid to potential differences in the CAL definition and the available methods of diagnosis.

Although CAL is sometimes defined as "a defect in the bowel wall at the anastomotic site, leading to communication of intra- and extraluminal compartments"^[17], this definition translates rather difficult to the clinical situation. Therefore, many authors formulate new definitions or diagnostic criteria in their studies, which usually include clinical and radiological features^[18], and the impact of a leak on the treatment plan. However, since the pathophysiology of anastomotic leakage is multifactorial, the manifestation of a clinical leak can be rather variable^[15]. Furthermore, due to the increased use of (routine) diagnostics such as CT or contrast enema, "radiological" leaks that do not eventually influence patient management are diagnosed more often. These factors complicate comparison of study results, and weaken the reliability of further analyses. This in turn hampers the construction of evidence-based guidelines on patient management and surgical technique.

Clearly, there is a need for a generally accepted and practical definition for CAL and its diagnostic criteria to serve as a template for future research on CAL and the clinical decision-making process^[19]. Several surveys have been performed to reach consensus regarding the definition of CAL, however, most of them were restricted to a single country^[19]. We hypothesized that the aforementioned reported differences in incidence rates between Asian and Western countries can partly be explained by differences in the definitions and diagnostic methods used. The aim of this study was therefore to determine the level of consensus regarding different aspects of a general definition of CAL within and between populations of Chinese and Dutch colorectal surgeons, that can be considered good representatives of the East and West, respectively.

MATERIALS AND METHODS

An online survey was performed among colorectal surgeons from the Netherlands and China. In the Netherlands, the survey was constructed and run through an online database using SurveyMonkey TM (Palo Alto, CA, United States). Colorectal surgeons in the Netherlands were identified from the contacts section of the colorectal subdivision of the Dutch Society of Gastro Intestinal Surgery (NVGIC): the Taskforce Coloproctology (WCP). Within this subdivision, 141 senior and junior colorectal surgeons were identified. Respondents were invited to partake in the online

Table 1 English questionnaire on definition of colorectal anastomotic leakage

General definition		
Do we have to consider the following findings as anastomotic leakage?	Yes	No
1 Extravasation of contrast after rectal enema on a CT scan		
2 Radiological collection around the anastomosis and no treatment		
3 Radiological collection around the anastomosis treated with antibiotics		
4 Radiological collection around the anastomosis treated with percutaneous drainage		
5 Abdominal sepsis and reoperation needed		
6 Necrosis of the anastomosis seen at reoperation		
7 Necrosis of the blind loop seen at reoperation		
8 Signs of peritonitis during reoperation		
9 Air bubbles around the anastomosis seen on a CT scan		
10 Free intra-abdominal air seen on a CT scan		
Clinical diagnosis		
In what extent do the following clinical parameters contribute to the suspicion of colorectal anastomotic leakage? Please note the relevance on a numeric scale of 0-10:		
1 Increased C-reactive protein		
2 Increased leukocytes		
3 Tachycardia		
4 Increased respiratory rate		
5 (Sub-) febrile temperature		
6 Postoperative ileus (> 4 d)		
7 Deterioration in clinical condition		
8 Abdominal pain, other than wound pain		
Radiological diagnosis		
Answer the following questions using percentages (0% = never, 100% = always)		
1 In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radiodiagnostics?		
2 In how many percent of patients with clinical suspicion of anastomotic leakage do radiodiagnostics change your treatment policy?		
3 In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage.		
4 In how many percent of cases do you consider a reoperation without previous radiodiagnostics?		
Early anastomotic leakage		
In your opinion, is 'very early (< 3 d) anastomotic leakage the result of technical failure?		
1 Yes		
2 No		

survey by email. Dutch surgeons completed the questionnaire between May and June 2015.

In China, the survey was conducted on the platform provided by DXY (www.dxy.cn), which is the largest medical website in China with more than one million registered medical users. An invitation was sent to all the registered colorectal surgeons to invite them to participate in a five-minute survey. Due to a relatively large number of registered users, the survey was designed to be terminated when 200 replies were received. Surgeons from Hong Kong, Macao, and Taiwan were not invited in this survey, because of the application of different medical systems in those areas. A demographic chart of the regions represented by the respondents can be observed in Figure 1.

The survey was divided into three major categories with questions addressing the general definition, and the clinical and radiological diagnosis of CAL. It was partly adapted from a previous study of Adams *et al.*^[20] and was initially constructed in English, and then translated to Dutch and Chinese by surgeons fluent in both English and Dutch and English and Chinese for the Dutch and Chinese versions, respectively, and checked for interpretation bias. Details of the English questionnaire are shown in Table 1 (See supplementary data for Dutch and Chinese versions).

Category I mainly focused on the agreement of general definitions used in the international literature^[20]. Surgeons were asked to state whether ten different clinical situations should or should not be included in a general definition. Category II focused on clinical manifestations and their predictive value for CAL. A 10-point grading scale ranging from 1 (not predictive at all) to 10 (very predictive) was used to assess the agreement of the respondents' views on the clinical parameters. The parameters used were partially adapted from the Dutch Leakage Score (DULK)^[11]. Category III consisted of four questions regarding the use of radiological examination and the influence of this diagnostic method on patient care. This third category was also partially adapted from Adams *et al.*^[20]. The last general question focused on surgeons' views regarding the cause of very early anastomotic leakage.

Definitions

Very early anastomotic leakage was defined as leakage occurring within the first three days post-surgery.

Postoperative ileus was defined as an interval of more than 4 d from surgery until passage of flatus or stool and the tolerance of an oral diet^[21].

Blind loop was defined as a bypassed loop of bowel after the construction of an end-to-end or end-to-side bowel anastomosis.

Statistical analysis

Basic descriptive statistics were used to summarize data for the online survey. Consensus was defined as > 80% agreement between respondents on various statements, as described by Duncan *et al.*^[22]. If less than 80% of respondents deemed the statements important, it was stated that no consensus was reached. Graphical depictions of information were used where appropriate to facilitate data interpretation. Chi square test or Mann-Whitney test were applied with proper indications. A *P*-value smaller than 0.05 was considered to indicate statistical significance.

RESULTS

Of the 141 colorectal Dutch surgeons who were invited to partake in the online survey, 62 respondents accepted the invitation, and 59 completed the survey, resulting in a 42% response rate and 95% survey completion. In total, 100% of 201 questionnaires



Figure 1 Demographic chart of the Chinese regions this survey covers. The gray scale reflects the number of participants in each region, varying from 21 from Zhejiang to 1 from Hainan. Correlating with the number of colorectal surgeons in each region, more surgeons from the east regions participated in this survey. Tibet and Ningxia had no participants, which also corresponds to the fact that the number of surgeons is very limited compared to the east provinces. Due to the application of different medical systems in these regions, Hong Kong, Macao, and Taiwan were not included in this survey.

received from Chinese surgeons were completed. A demographic chart of the regions represented by the respondents can be observed in Figure 1, as it shows that this survey covers 96.8% (30/31, Hong Kong, Macao, Taiwan not included) of provinces and areas of China.

Consensus was found on only one clinical situation proposed as an element of a general definition in both countries: 'extravasation of contrast on enema' (Figure 2), and in the Netherlands on two additional elements: radiological collection for which percutaneous drainage was needed (50/59 respondents, 85%) and necrosis of the anastomosis visible upon reintervention (51/59 respondents, 86%). For all other items on the available general definitions, clinical and radiological diagnosis of CAL, no consensus was found. Scores were significantly different between China and the Netherlands for the following elements: radiological collection treated conservatively (21% vs 39%, respectively, $P = 0.010$), necrosis of the blind loop on reintervention (41% vs 69%, respectively, $P \leq 0.001$), and air surrounding the anastomosis on a CT scan (65% vs 44%, respectively, $P = 0.004$).

Grades given for the clinical parameters are shown in Figure 3 for both China and the Netherlands. Clinical deterioration, increased C-reactive protein (CRP), tachypnea, and tachycardia were seen as being most contributory for the clinical suspicion of CAL in the Netherlands, and were given a weighed score of 7.83, 7.45, 7.13 and 7.13, respectively (Table 2). In China, clinical deterioration and abdominal pain other than wound pain were deemed most attributable for the suspicion of anastomotic leakage in the direct postoperative period, with scores of 6.67 and 6.61, respectively. Increased plasma concentration of CRP received the lowest score of all parameters in China (4.35), while in the Netherlands this was deemed

Table 2 Sensitivity scores of clinical parameters for the suspicion of anastomotic leakage in the direct postoperative period in China and The Netherlands

Clinical parameter	China	The Netherlands	<i>P</i> -value
	Score \pm SD	Score \pm SD	
Increased CRP	4.35 \pm 2.466	7.45 \pm 1.871	< 0.001
Leukocytosis	5.96 \pm 2.596	6.53 \pm 1.824	0.095
Tachycardia	4.55 \pm 2.411	7.13 \pm 1.937	< 0.001
Tachypnea	4.46 \pm 2.244	7.13 \pm 1.937	< 0.001
Febrile temperature	6.23 \pm 2.281	5.86 \pm 1.963	0.207
Postoperative ileus	4.47 \pm 2.363	5.76 \pm 1.679	< 0.001
Clinical deterioration	6.67 \pm 2.033	7.83 \pm 1.205	< 0.001
Abdominal pain	6.61 \pm 2.247	6.74 \pm 1.835	0.659

CRP: C-reactive protein.

more sensitive (7.45, $P < 0.001$). Upon categorization of the grades for the value of clinical parameters into different categories of the numeric scale: disagree (0-3), neutral (4-6) and agree (7-10), most surgeons from both countries (45%-59% of surgeons for each parameter) remained neutral towards the added value of specific clinical parameters during the postoperative course.

The data on radiodiagnostics are shown in Table 3. The majority of Chinese and Dutch surgeons perform radiodiagnostics upon clinical suspicion of a leak. The distribution of the answers over the different classifications, however, was significantly different between the two nationalities (Chi square test, $P = 0.020$). Expected false-negative rates for CT scans were equal for surgeons in both countries. A significantly larger portion of the Chinese colorectal surgeons (25.4% vs 13.6%, $P \leq 0.001$) would consider performing a reoperation for the suspicion of CAL without performing radiological diagnostics. The distribution of the scores differed significantly between countries as to in how many cases a reoperation is considered without previous radiodiagnostics (Chi square test, $P = 0.002$).

Concerning the question about early CAL, 90.6% of the Chinese surgeons agreed that the cause of such should be considered a technical failure, whilst only 70.4% of the Dutch colorectal surgeons agreed to this statement ($P \leq 0.001$).

DISCUSSION

Despite extensive research in the field of CAL, no international consensus regarding a practical definition exists, which limits the transparency and comparison of study outcomes. Several definitions of CAL have been proposed during the last decade^[18,23], but review of the literature shows that newly published papers fail to adopt these definitions^[24]. Instead, authors seem to prefer to use their own definitions or no definition at all^[24]. It could be postulated that these previously proposed definitions were not yet implemented in clinical practice and (retrospective) research because of limited awareness of the existence of such a

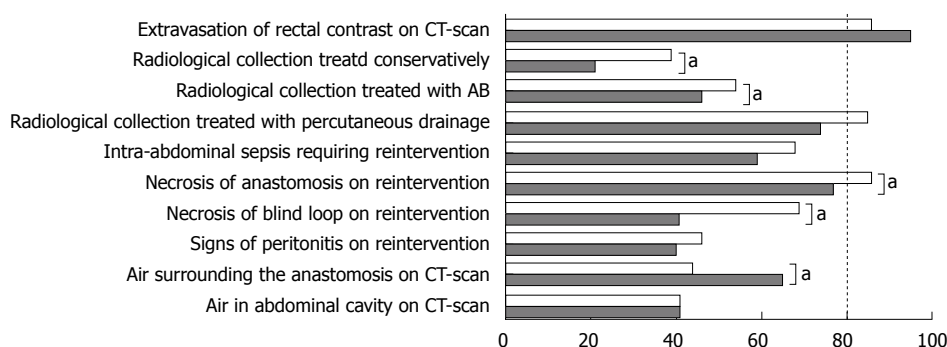


Figure 2 Percentage of respondents in agreement to general definitions of colorectal anastomotic leakage in the Netherlands (white bars) and China (dark grey bars). The dotted line indicates the 80% consensus threshold for the different statements. An a indicates a significant ($P < 0.05$) difference between percentages of agreement of Dutch and Chinese surgeons.

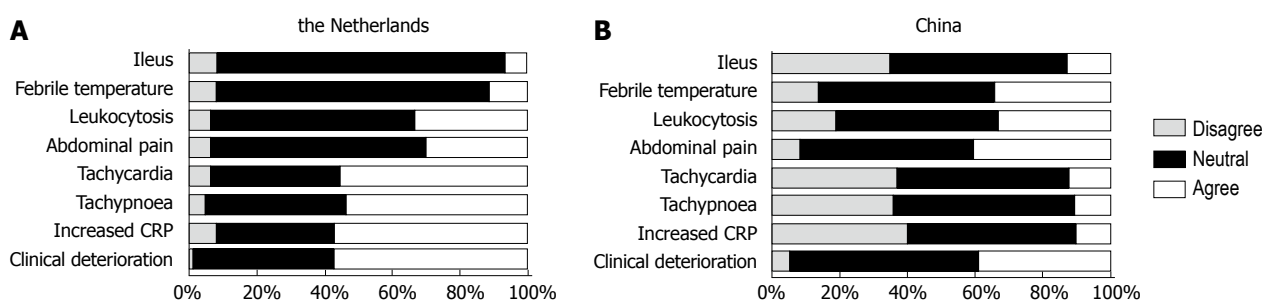


Figure 3 Distribution of categorized scores for the value clinical parameters in the direct postoperative phase. A: Comparison between the Netherlands and China. B: Scores are divided into three categories: numeric scales ranging from 0-3 are depicted in grey (disagree), 4-6 depicted in black (neutral), and numeric scales ranging from 7-10 (agree) in white.

definition and/or lack of support from a large expert group.

Reports from Asian studies show CAL rates that are substantially lower than those reported by Western research groups^[25]. This could partially be explained by demographic differences that exist in patient population, availability and use of diagnostic tools, or how perioperative care is structured. Another explanation could be that Chinese surgeons only report a leak as such when reintervention is required.

On the other hand, despite the lower prevalence of obesity in Asian countries, rates of type II diabetes mellitus and metabolic syndrome are relatively high due to ethnic and genetic factors. Indeed, Asians account for 60% of diabetes mellitus cases worldwide^[26], which is considered an important risk factor for CAL^[27,28]. Furthermore, the most common location of colorectal tumors in the Asian population is the left hemicolon^[16,29], compared to the Western population, in which the predominant side is the right^[29,30]. The literature shows a significantly higher CAL risk for surgeries on colorectal tumors located in the left hemicolon^[31]. These regional differences therefore fail to completely explain the variation in reported CAL rates. It is very likely that important regional differences exist as to what is considered an anastomotic leak, *i.e.*, Asian surgeons may report a leak mostly when a reintervention is required, while the Western surgeons may report latent leaks. In order to gain more insight into these differences in views, the present survey was conducted

both in China and in the Netherlands, countries that are considered to be representative for their continent.

In the first part of the survey, surgeons were asked whether different statements including clinical and radiological signs and interventions regarding CAL should be considered anastomotic leakage. Of ten statements, only one was deemed as CAL by more than 80% of respondents in both countries: 'Extravasation of contrast after rectal enema visible on a CT scan'. This is generally considered a radiological hallmark sign for anastomotic leakage after left-sided colorectal surgery and should naturally be included in a general definition. Moreover, other important and evident CAL signs including "Radiological collection around the anastomosis treated with percutaneous drainage" and "Necrosis of the anastomosis seen at reoperation" received more than 80% positive responses in the Netherlands, however, not in China, and thus were not considered as consent according to the predetermined criteria. Despite this, the majority of the Chinese surgeons also agreed on these items and their answers did not differ significantly from those of their Dutch colleagues. In conclusion, it seems that for the evident signs of CAL, the majority of surgeons from both countries have quite similar views.

"Radiological collection treated conservatively" was only considered to be CAL in 21% of the Chinese surgeons (versus 39% in the Netherlands), which is almost a consensus of NOT including this statement in a general definition of CAL. On the contrary, it is at

Table 3 Surgeons' opinion regarding the value of radiodiagnostics in the diagnosis of colorectal anastomotic leakage

Responders, <i>n</i> (%)	China (%)	The Netherlands (%)	<i>P</i> -value
In how many percent of patients with clinical suspicion of anastomotic leakage do you perform radiodiagnostics?			
	202 (100)	55 (93)	
0%-20%	3.0	0	
21%-40%	6.4	0	
41%-60%	6.9	1.8	
61%-80%	24.3	16.4	
81%-100%	59.4	81.8	
Average	83.3	91.5	0.285
In how many percent of patients with clinical suspicion of anastomotic leakage do radiodiagnostics change your treatment policy?			
	202 (100)	54 (91.5)	
0%-20%	10.9	13.0	
21%-40%	9.9	5.6	
41%-60%	27.7	44.4	
61%-80%	30.2	25.9	
81%-100%	26.7	11.1	
Average	63.6	55.9	0.028
In how many cases did the CT scan report no anastomotic leakage while there finally was an anastomotic leakage?			
	202 (100)	52 (88.1)	
0%-20%	40.6	51.9	
21%-40%	29.2	28.8	
41%-60%	25.2	15.4	
61%-80%	4.0	1.9	
81%-100%	1.0	1.9	
Average	31.8	28.7	0.221
In how many percent of cases do you consider a reoperation without previous radiodiagnostics?			
	202 (100)	53 (89.8)	
0%-20%	58.4	84.9	
21%-40%	18.8	13.2	
41%-60%	17.3	0	
61%-80%	4.5	0	
81%-100%	1.0	1.9	
Average	25.4	13.6	< 0.001

least remarkable that in current grading systems, a radiological collection can be considered anastomotic leakage. As such this is reported as a grade A CAL according to the International Study group of Rectal Cancer (ISREC)^[18] and grade I - II CAL according the Clavien-Dindo Scale.

Despite the fact that only a minority of Dutch surgeons consider conservatively treated radiological collections as CAL, these numbers are higher than those among the Chinese. These differences in views regarding the subclinical signs of CAL may eventually lead to a significantly higher reported CAL rate in the Dutch studies than the Chinese ones. However, considering the fact that more than 30%^[32] of the CAL do not require invasive intervention, the treatment provided by surgeons from both countries may eventually be similar, *i.e.*, leading to a similar intervention rate for the complication. To rule out the reporting difference in this regard, one solution is to report complications with a Clavien-Dindo score higher than IIIa, which actually is also commonly accepted and applied in recent studies.

The second part of the survey focused mainly on

clinical markers and parameters for CAL. Early clinical diagnosis of CAL remains a challenge for surgeons worldwide. Many clinical symptoms and biomarkers have been suggested as early signs of CAL^[33-36]. However, previous studies of these parameters have shown that almost none of these parameters yield sufficient diagnostic accuracy to allow for a confirmative diagnosis^[37]. This explains our findings that most surgeons do not base their diagnosis of CAL on these parameters, which results in a relatively low score of their contribution to the suspicion of CAL. Surgeons from both countries deemed "deterioration of clinical condition" as an important symptom of CAL, which further accentuates the complexity of CAL diagnosis based on its clinical manifestations. We believe the surgeons' opinions indeed reflect the unsatisfactory status of CAL diagnosis, which stresses the need for further research in this field^[38]. However, important differences exist between the two countries. Although surgeons from both countries agreed about the predictive value of higher temperature, abdominal pain other than wound pain, and increased leukocyte count, more than half of the clinical parameters scored significantly lower in China than in the Netherlands. Although these abnormal clinical manifestations are indeed very common after gastrointestinal surgery^[39], it seems that they are considered less suggestive by the Chinese surgeons.

The third part of the survey focused on radiological tools used in the diagnosis of CAL. Based on the present data, the majority of the surgeons in both countries would perform radiological examination on patients in whom CAL was suspected (these numbers are slightly higher in the Netherlands), and more than half of the treatment plans would be changed after the imaging. In this regard, although differences have been found in the views of Chinese and Dutch surgeons regarding the definition of CAL, the treatment they provide is similar. However, our data also show that surgeons from both countries do not blindly rely on the results from radiodiagnostics. Instead, they state that in approximately 30% of the cases in which CAL is suspected, CAL is eventually diagnosed in spite of a negative radiological report. This correlates with previously reported false negative rates of CT scans^[40]. Experience with inaccurate CT scan reports may be a reason for surgeons to consider reoperation without affirmative CT results, which according to the data, occurs in about 25% of cases.

Further research and education may facilitate the achievement of international consensus. However, definition without considerations of the practical issues in different regions is unlikely to gain sufficient popularity. In 2010, the ISREC proposed a graded system for the diagnosis and treatment of CAL^[18]. Grade A CAL refers to anastomotic leakage for which no active therapeutic intervention is required. It seems that this grade correlates with the second statement "Radiological collection surrounding the anastomosis

treated conservatively” that is not classified as CAL according to the majority of both Dutch and Chinese surgeons. This discrepancy between an established definition and the views of colorectal surgeons could partly explain why the ISREC definition has not been adopted in practice and science. In accordance with that, our survey clearly demonstrates how different practices may influence surgeons’ opinion.

For example, in the Netherlands the Enhanced Recovery After Surgery (ERAS) program has been widely adopted for years, and recommends no abdominal drainage after surgery. In China, on the contrary, ERAS is less commonly implemented, and an intra-abdominal drain is often left *in situ* for longer periods after surgery. Moreover, CT imaging is less commonly used as radiodiagnostic for CAL, and laboratory analysis by means of CRP is not yet implemented in routine practice in many rural areas. This could explain why increased CRP was deemed least contributory in the diagnostic process in the present survey. These points, though small, significantly influenced the results, and would certainly impact the applicability of a proposed CAL definition.

To successfully embed a definition in clinical practice, research on CAL would greatly benefit from establishing a uniform definition and recording in national databases. We will therefore continue to perform an extensive and systematic literature review. The results from that review and the consensus assessment described in this paper will lead to an international Delphi analysis that will allow us to reach consensus on a new definition proposal that will be supported by a large panel of experts. We sincerely welcome others to participate in this further research, in order to formulate a new definition based on joint experience and opinions.

The most important limitations of the study are the following. The content of questionnaires is always susceptible to researcher imposition and there may be a level of subjectivity in the answers given. Furthermore, the relatively low numbers of respondents from both countries would have a negative influence of the generalizability of study results. Finally, the original questionnaire was constructed in English and translated into Dutch and Chinese, which could introduce bias and weaken the validity of comparisons between the countries. Finally, as some of the clinical parameters used in the questionnaire were derived from the DULK-score, which was constructed and validated in the Netherlands, it is plausible that the Dutch participants scored similarly on these items because they were familiar with the content of the DULK-score, because they have been (in)directly involved in the construction of the scoring system. However, the use of the DULK-score has not remained limited to the Netherlands, and it is unknown whether the subset of Dutch surgeons familiar with the DULK-score is higher than the number of Chinese surgeons who use this score routinely, and whether this difference is large enough to alter the data

significantly.

In conclusion, no international consensus of a practical definition of CAL is yet available, which limits the transparency and comparison of published results. The present international online survey proves the inconsistent views as to what is considered CAL among surgeons in the Netherlands and China, and shows large differences between countries. Dutch surgeons are more likely to report ‘subclinical’ leaks as CAL, which partly explains the higher reported Dutch CAL rates. Surgeons from both countries rely on radiological diagnostics and laboratory parameters in the decision-making process, but are well aware of the limitations of these diagnostic aids. A Delphi analysis within a representative panel of colorectal surgeons is desired to develop a widely accepted definition of CAL.

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COMMENTS

Background

Colorectal anastomotic leakage (CAL) is the most feared complication after colorectal surgery. No international consensus exists regarding a general definition of CAL.

Research frontiers

Over the past decades, thousands of articles on CAL have been published. Unfortunately, a uniform and accepted worldwide definition of CAL is not available. This limits the transparency and comparison of study results and usefulness in clinical practice.

Innovations and breakthroughs

An international survey has been performed to identify the differences in reported definitions of CAL and to evaluate the opinions of expert leaders in both a Western and Eastern country.

Applications

The present international online survey proves the inconsistent views as to what is considered CAL among surgeons in the Netherlands and China, and shows large differences between the countries. This is in line with the current literature, since there is no uniform accepted definition worldwide. We therefore propose to perform a systematic literature review to identify the available definitions. The final stage is to perform a Delphi analysis within a representative panel of colorectal surgeons to develop a widely accepted definition of CAL.

Terminology

CAL is the major complication after colorectal surgery with a stable incidence (1.5%-23%). It is associated with high rates of morbidity and mortality, poor quality of life, and increased health expenditure. Since CAL influences the direct postoperative course and has recently been proven to impact oncological outcome as well, it is frequently used as an outcome measure in clinical studies.

Peer-review

In this study, the authors have presented a thorough and critical analysis of the availability of a definition of CAL and the opinions of both Dutch and Chinese surgeons regarding this definition.

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How to treat intestinal obstruction due to malignant recurrence after Whipple's resection for pancreatic head cancer: Description of 2 new endoscopic techniques

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Abstract

The prognosis of pancreatic cancer remains poor, even after initial surgical therapy. Local recurrence after Whipple's pancreaticoduodenectomy may lead to intestinal obstruction at the level of the afferent limb or the alimentary limb. Endoscopic insertion of a self-expandable metal stent (SEMS) into the intestinal malignant stricture is the preferred method of choice for palliation. We describe two new endoscopic techniques to treat a malignant intestinal obstruction with the insertion of a SEMS into the afferent limb and the alimentary limb. A case of malignant gastric outlet obstruction after a Whipple's resection was treated by the creation of an endoscopic gastrojejunostomy by the insertion of a lumen apposing HotAxios stent in between the stomach and the alimentary limb under fluoroscopic and endoscopic ultrasound control. Biliary obstruction and jaundice caused by a malignant stricture of the afferent limb after a Roux-en-Y Whipple's resection was treated by the insertion of a SEMS by means of the single-balloon overtube-assisted technique under fluoroscopic control. Feasibility and advantages of both techniques are discussed.

Key words: Intestinal obstruction; Self-expandable metal stent; Whipple's pancreaticoduodenectomy

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Core tip: Malignant recurrence after Whipple's pancreatico-duodenectomy is frequent and may lead to intestinal obstruction. Endoscopic palliation of the intestinal obstruction is challenging. We present 2 endoscopic techniques: Endoscopic ultrasound-guided creation of a gastrojejunostomy using a fully covered lumen apposing metal stent, and insertion of a self-expandable metal stent by means of the single-balloon overtube-assisted technique under fluoroscopic control.

Mouradides C, Taha A, Borbath I, Deprez PH, Moreels TG. How to treat intestinal obstruction due to malignant recurrence after Whipple's resection for pancreatic head cancer : Description of 2 new endoscopic techniques. *World J Gastroenterol* 2017; 23(33): 6181-6186 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6181.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6181>

INTRODUCTION

Whipple's pancreatico-duodenectomy was named after Alan Oldfather Whipple who described the technique in 1935^[1]. It still is the surgical treatment of choice to cure pancreatic head cancer^[2]. Modifications of the technique have emerged, including pylorus-preserving Whipple's resection and Roux-en-Y intestinal reconstruction^[3]. After resection of the pancreatic head, both the remnant bile duct and pancreatic duct have to be anastomosed to the afferent limb (hepaticojejunostomy/pancreaticojejunostomy) and food passes through the alimentary limb. Despite the different surgical Whipple's variations, the risk of local tumor recurrence after Whipple's resection for pancreatic head cancer remains more than 50%^[4]. Few cases present with malignant intestinal obstruction at the level of the afferent or the alimentary limb. Endoscopic palliative therapy by means of insertion of self-expandable metal stents (SEMS) is the preferred method of choice to treat malignant intestinal obstruction in case of pancreatic cancer^[2]. However, endoscopic SEMS placement in the afferent or the alimentary limb remains a challenging procedure^[5]. We report on the endoscopic treatment modalities of two cases of malignant intestinal obstruction due to tumor recurrence after Whipple's pancreatico-duodenectomy.

CASE REPORT

Case 1

A 71 year-old woman was referred with symptoms of upper gastrointestinal obstruction because of local tumor recurrence at the level of the gastrojejunal anastomosis with peritoneal carcinomatosis. She underwent Whipple's resection for pancreatic head



Figure 1 Coronal computed tomography image of a non-covered metallic stent with peroral contrast in the dilated afferent limb in a patient with classical Whipple's duodenopancreatectomy. Note the obstructed empty alimentary limb underneath the stomach (arrows).



Figure 2 Linear endoscopic ultrasound image of the transgastric puncture of the obstructed alimentary limb (arrow) with a 19 gauge needle.

cancer two years earlier. Initially, an uncovered enteral SEMS (Wallflex 60 x 22 mm, Boston Scientific) was inserted and ended up in the afferent limb, eventually obstructing the alimentary limb worsening the gastric outlet syndrome. CT scan confirmed the dilated afferent limb towards the hepaticojejunostomy, with food remnants and the empty alimentary limb underneath the stomach (Figure 1). Since the uncovered SEMS could not be removed from the afferent limb because of tumor ingrowth obstructing the entrance to the alimentary limb, we decided to create an endoscopic gastrojejunostomy, using the HotAxios device (XLumina Axios 10 mm x 15 mm, Boston Scientific), under endoscopic ultrasound (EUS) guidance (linear-array echoendoscope GF-UCT 180, Olympus) and fluoroscopy. The HotAxios device is a cystotome catheter with a preloaded covered lumen apposing metal stent. It is developed to drain pancreatic pseudocysts with an all-in-one device^[6]. Here we used it to create an endoscopic gastrojejunostomy. The empty alimentary limb was identified by transgastric EUS, accessed with a 19 gauge needle (Figure 2), and confirmed fluoroscopically by intraluminal contrast dye injection.

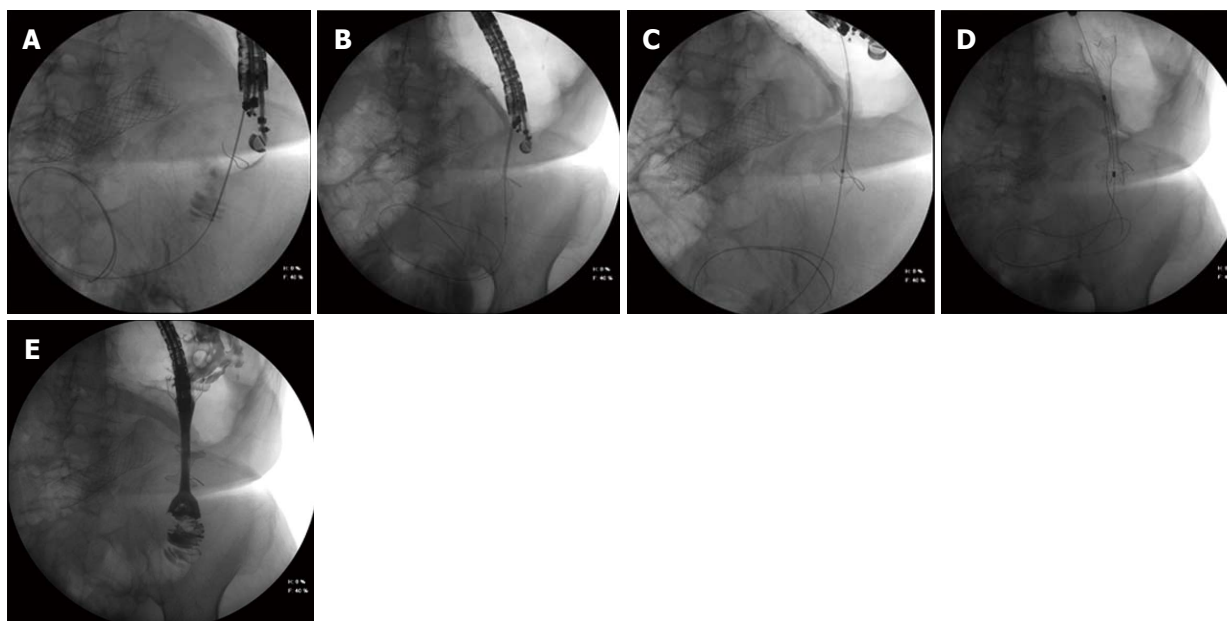


Figure 3 Fluoroscopic image. A: The transgastric access of the alimentary limb using a 19 gauge needle. Correct access of the limb was confirmed after injection of contrast dye. A: 0.035 inch guidewire is introduced into the alimentary limb. Note the SEMS in the afferent limb; B: The HotAxios lumen apposing stent deployment between the alimentary limb and the gastric lumen; C: The HotAxios lumen apposing stent migrating outside to stomach. The distal flange is well positioned inside the lumen of the alimentary limb; D: The fully covered oesophageal stent inside the HotAxios lumen apposing stent, creating a gastrojejunostomy; E: Fluoroscopic image confirming the creation of a gastrojejunostomy between the stomach and the alimentary limb using the combination of a HotAxios lumen apposing stent and a fully covered oesophageal stent. Water soluble contrast dye is injected through the oesophageal stent into the alimentary limb. Note the non-covered SEMS in the afferent limb.

Next, a 0.035 inch guidewire was placed (Figure 3A), allowing smooth introduction of the HotAxios stent deployment system into the alimentary limb using electrocautery. The fully covered 10 mm HotAxios lumen apposing stent was deployed under fluoroscopic and endoscopic guidance, with the distal flange in the intestinal lumen, and the proximal flange in the stomach (Figure 3B). However, upon release, it immediately migrated outside the stomach because of the distance between the alimentary limb and the stomach (Figure 3C). A fully covered metal esophageal stent (Taewoong Medical Niti, 80 mm x 22 mm) was introduced over the guidewire and deployed inside the HotAxios stent, with the proximal part protruding into the gastric lumen (Figures 3D and E). After the procedure, the patient quickly resumed oral feeding.

Three months later, she was readmitted for vomiting due to intragastric migration of the esophageal stent. The HotAxios stent was still in place. A fully covered Nagi stent (Taewoong Medical, 20 mm x 16 mm) was placed inside the HotAxios stent, and the patient resumed oral feeding. Seven months after the initial presentation, she died due to malignant progression of the disease.

This case illustrates the feasibility of the HotAxios system in creating an EUS-guided gastrojejunostomy to treat malignant gastric outlet syndrome. This technique has recently emerged as an alternative to surgical gastrojejunostomy treating benign and malignant gastric outlet syndrome, with technical success rate

of 92% and 85% clinical success^[7-9]. Serious adverse events of this endoscopic ultrasound technique were reported in 11.5%, and encompass gastrointestinal perforation, peritonitis and bleeding^[9]. We encountered another complication. After deployment of the HotAxios lumen apposing stent to create the gastrojejunostomy, the gastric flange migrated outside the stomach without the possibility to reposition it into the stomach. Since the guidewire was still in place, we were able to save the gastrojejunostomy by inserting a second fully covered oesophageal SEMS inside the HotAxios stent.

Case 2

A 76-year-old woman who underwent Roux-en-Y Whipple's resection two years earlier for pancreatic head cancer, was referred because of obstructive jaundice and dilated intrahepatic bile ducts. Percutaneous transhepatic cholangiography and biliary drainage excluded an obstruction at the level of the bilioenteric anastomosis. However, afferent limb syndrome was diagnosed based on the obstruction of the afferent limb 20 cm distally from the hepaticojejunostomy with a dilated intestinal segment between the hepaticojejunostomy and the enteral stricture of the afferent limb (Figure 4A). Next, we performed a single-balloon enteroscopy (SBE) through the Roux-en-Y anastomosis into the afferent limb under fluoroscopic guidance, and reached the jejunal stenosis, with endoscopic characteristics of a malignant stricture (Figure 5). The length of the

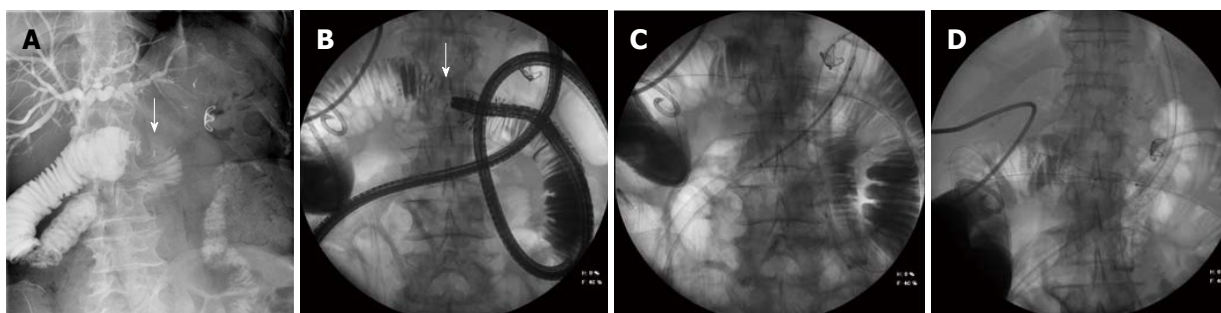


Figure 4 Fluoroscopic image. A: A percutaneous transhepatic cholangiography confirming patency of the biliary anastomosis. The malignant stricture (arrow) is located at the level of the afferent limb (afferent limb syndrome), leading to dilated intrahepatic bile ducts and a dilatation of the afferent limb. Note the presence of an old Ovesco clip in the stomach. B: The single-balloon enteroscopy up to the level of the malignant stricture in the afferent limb. Contrast injection through the working channel of the enteroscope clearly delineates the length and the malignant aspect of the stricture (arrow). C: The peroral insertion technique of the self-expandable metal stent over a stiff guidewire through the overtube with the balloon inflated to protect the intestinal mucosa and to guide the catheter up to the malignant stricture of the afferent limb. D: The self-expandable metal stent in place in the malignant stricture of the afferent limb. Both the stiff guidewire and the overtube of the single-balloon enteroscope were used to insert the SEMS. SEMS: Self-expandable metal stent.

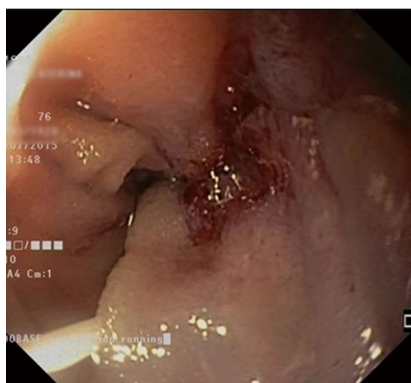


Figure 5 Endoscopic image of the malignant stricture of the afferent limb. Note the guidewire inserted into the stricture.

stricture was estimated by means of the injection of water soluble contrast. A 0.035 inch guidewire was inserted into the stricture under endoscopic and fluoroscopic control, and a CRE dilation balloon (8-10 mm) was inserted over the guide wire to dilate the stricture. Biopsies confirmed the intestinal recurrence of a pancreas adenocarcinoma. After a multidisciplinary oncology discussion, a non-surgical endoscopic approach was suggested. One week later, a second SBE was performed in order to place a SEMS into the malignant stricture. Again, the length of the stricture was estimated by contrast injection under fluoroscopic control (Figure 4B). A 0.035 inch stiff guidewire (VisiGlide 450 cm, Olympus) was inserted into the dilated jejunal segment proximal of the stricture. The overtube of the SBE was anchored as close as possible near the malignant stricture with the balloon inflated. Next, the enteroscope was removed under fluoroscopic guidance, leaving the guidewire and the overtube in place. An uncovered SEMS (Wallflex 60 mm x 22 mm, Boston Scientific) was inserted over the guidewire through the overtube into the stricture, located by means of the previously injected contrast

(Figure 4C). The stent was deployed under fluoroscopic guidance (Figure 4D). Afterwards, the enteroscope was reintroduced through the overtube to control for the correct positioning of the SEMS. The percutaneous biliary drain was removed and the patient was discharged two days later with normal oral food intake. Gemcitabine-based chemotherapy was started.

Three months later, she presented again with obstructive jaundice and afferent limb syndrome suggestive of tumor invasion of the uncovered SEMS. A third SBE procedure was performed, confirming the diagnosis of SEMS obstruction. We used the same single-balloon overtube-assisted technique to insert a new partially covered SEMS (ComVi Enteral Colonic Stent 100 mm x 22 mm, Taewoong) inside the uncovered SEMS. No complications were encountered. Ten months after the second SEMS insertion, she died due to malignant progression of the disease.

This case emphasizes the usefulness, feasibility and reproducibility of the single-balloon overtube-assisted technique for SEMS insertion in malignant strictures of the small intestine, especially in patients with altered anatomy such as Roux-en-Y anastomosis where conventional endoscopes cannot reach. Since the currently available enteral SEMS deployment catheters cannot be used through the working channel (2.8 or 3.2 mm) of the 200 cm long enteroscope, the SEMS is introduced over a long stiff guidewire through the balloon-anchored overtube under fluoroscopic control. This balloon-assisted overtube technique enables to relieve intestinal obstruction without surgical intervention by a combined endoscopic and fluoroscopic approach^[10,11].

DISCUSSION

Malignant recurrence after (modified) Whipple's pancreatico-duodenectomy for pancreatic head adenocarcinoma remains high^[4]. Sometimes, local

recurrence may lead to the afferent limb syndrome (stricture of the afferent limb) or a gastric outlet-like syndrome (stricture of the alimentary limb) because of intestinal tumor infiltration. In the case of malignant intestinal obstruction following Whipple's resection, palliative surgery is generally indicated to obtain symptom relief (resection of an intestinal segment, enterostomy or intestinal bypass)^[12]. Case reports have shown that endoscopic enteral SEMS placement has become feasible, either by the creation of an EUS-guided gastrojejunostomy or by insertion of a SEMS by means of the balloon-assisted overtube technique^[7-11]. We illustrated both procedures in two cases of malignant intestinal obstruction (afferent and alimentary limb obstruction) after Whipple's resection.

COMMENTS

Case characteristics

Both patients underwent Whipple's pancreaticoduodenectomy as a curative treatment of pancreatic head cancer. However, local tumor recurrence in the alimentary limb resulted in the gastric outlet syndrome (case 1) and local tumor recurrence in the afferent limb resulted in obstructive jaundice (case 2).

Clinical diagnosis

The patient with the gastric outlet syndrome presented with recurrent vomiting and weight loss (case 1). The patient with the afferent limb syndrome presented with fever, abdominal pain and jaundice.

Differential diagnosis

Vomiting may be a side-effect of chemotherapy (gemcitabine) or may be caused by peptic ulcer disease (case 1). Obstructive jaundice may be the clinical sign of a postoperative stricture at the level of the hepaticojejunostomy (case 2).

Laboratory diagnosis

The patient with the gastric outlet syndrome presented a slight increase in CA19.9 tumor marker (case 1). The patient with the afferent limb syndrome presented with elevated CRP and liver function tests (case 2).

Imaging diagnosis

Abdominal CT scan of the patient with the gastric outlet syndrome suspected peritoneal carcinomatosis (case 1). MRI-MRPC of the patient with the afferent limb syndrome suspected a postoperative stenosis at the level of the hepaticojejunostomy (case 2).

Pathological diagnosis

No histological biopsies were taken during the endoscopic ultrasound (EUS)-guided gastrojejunostomy procedure to confirm local tumor recurrence (case 1). During the first single-balloon enteroscopy biopsies were taken at the level of the afferent limb stricture, confirming the presence of a well-differentiated pancreatic adenocarcinoma (case 2).

Treatment

Both patients were treated endoscopically in a palliative setting. The first patient underwent EUS-guided gastrojejunostomy followed by chemotherapy (Oxaliplatin) (case 1). The second patient underwent self-expandable metal stent (SEMS) insertion into the afferent limb followed by chemotherapy (Gemcitabine) (case 2).

Related reports

The two endoscopic techniques presented here have been described previously

under the form a case reports.

Term explanation

Whipple's resection is the surgical curative treatment for pancreatic head cancer, with resection of the pancreatic head and the duodenum and the creation of a hepaticojejunostomy and a pancreaticojejunostomy on to the afferent jejunal limb. The afferent limb syndrome is caused by an obstruction at the level of the afferent limb after Whipple's resection. The gastric outlet syndrome is caused by an obstruction at the level of the alimentary limb after Whipple's resection.

Experiences and lessons

Local tumor recurrence remains high after initial curative Whipple's pancreaticoduodenectomy for pancreatic head cancer. The clinical presentation as the gastric outlet syndrome or the afferent limb syndrome is rare. New endoscopic techniques may allow to overcome these clinical syndromes by creating an EUS-guided gastrojejunostomy or by SEMS insertion into the afferent limb.

Peer-review

This is a well-written and illustrated overview of two endoscopic techniques for the palliation of intestinal obstruction due to local tumor recurrence after Whipple's pancreaticoduodenectomy for pancreatic head cancer.

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Arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer

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Informed consent statement: Written informed consent was obtained from the patient for publication of this case report and any accompanying images.

Conflict-of-interest statement: The authors declare no conflict-of-interest.

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Abstract

Arterioportal shunt (APS) is an organic communication between the hepatic arterial system and the portal venous system. The APS is one of the major causes of transient hepatic attenuation differences on dynamic computed tomography (CT) or magnetic resonance imaging (MRI). This condition is usually associated with trauma, liver cirrhosis, and malignancies of the liver. However, there has been no report about oxaliplatin-induced APS. A 41-year-old male was diagnosed with Stage III B gastric cancer. The patient initially underwent neoadjuvant chemotherapy with capecitabine and oxaliplatin. After 3 cycles of therapy, the mass had markedly decreased, and a total gastrectomy with splenectomy was performed. Since the malignancy was locally invasive, the patient was continued on the same regimen of the adjuvant chemotherapy. After 3 more cycles, a computed tomography revealed a 1 cm sized arterial-enhancing nodule in the right lobe of the liver. An MRI revealed an arterial enhancing lesion, and a positron emission tomography CT scan showed a hypermetabolic lesion in the same portion of the liver. We tried to perform a liver biopsy; however, an ultrasonography could not detect any mass. A presumptive diagnosis of an APS due to a recurrent cancer was made. We found a similar but slightly different case report of an oxaliplatin-induced liver injury, mimicking a metastatic tumor on an MRI. Based on a prior report, the patient was continued

on treatment with adjuvant chemotherapy following discontinuation of oxaliplatin. After 2 cycles, the arterial enhancing liver mass resolved, supporting the final diagnosis of an APS, related to oxaliplatin-induced sinusoidal injury. The patient has not experienced any a relapse after two years of additional follow up recurrent gastric cancer upon interpretation of multiple imaging modalities.

Key words: Liver; Arterioportal shunt; Recurred cancer; Oxaliplatin; Transient hepatic attenuation differences

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Core tip: Although there have been several recent reports about oxaliplatin-induced sinusoidal injury, this is first case report of a non-tumorous incidental arterioportal shunt following oxaliplatin chemotherapy. We made a presumptive diagnosis of an AP shunt mimicking a recurrent gastric cancer, due to an oxaliplatin-induced transsinusoidal injury. This case is the first report of an oxaliplatin-induced incidental arterioportal shunt mimicking recurrent gastric cancer on various images.

Kim HB, Park SG. Arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer. *World J Gastroenterol* 2017; 23(33): 6187-6193. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6187.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6187>

INTRODUCTION

Transient hepatic attenuation differences (THAD) lesions indicate areas of parenchymal enhancement, visible during the hepatic artery phase on a dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and are thought to be a physiological phenomenon due to the dual hepatic blood supply. The arterioportal shunt (APS) is one of the most important THAD^[1,2].

APS is an organic communication between the hepatic arterial system and the portal venous system, and is caused by the redistribution of arterial flow into a focal region of the portal venous flow. This condition is associated with hepatic tumors, trauma, intervention, liver cirrhosis and metastatic tumors^[2-5].

CASE REPORT

A 41-year-old Korean man was admitted with a chief complaint of epigastric pain. The patient initially underwent an endoscopy, which revealed a 4 cm sized ulcerofungating mass in the gastric cardia and CLO test (Campylobacter-like organism test; Rapid

urease test) was negative. Pathologic findings revealed poorly differentiated adenocarcinoma without intestinal metaplasia and a CT scan showed a bulky ulcerative mass with direct invasion of the pancreas, and the left diaphragm. Furthermore, there were multiple perigastric and upper aortic lymph node enlargements. Based on these findings, the patient was diagnosed with Stage III B gastric cancer (T4N2M0) (Figure 1A and B). He had a far advanced stage of gastric cancer; therefore, the patient underwent firstly a neoadjuvant chemotherapy with capecitabine and oxaliplatin. After 3 cycles of this therapy (#1-#3), the mass had markedly decreased and a total gastrectomy with splenectomy was performed (Figure 1C and D). Since this was a stage III B gastric cancer [T4bN0 (0/48) M0], the patient was continued on the same regimen as adjuvant chemotherapy

After another 3 cycles of therapy (#4-#6), a follow-up CT demonstrated an arterial enhancing mass like lesion in the peripheral portion of the right lobe of the liver (Figure 2).

We presumed initially a new hepatocellular carcinoma or recurrent gastric cancer, and we evaluated the arterial enhancing mass like lesion. He had a no history of hepatitis (hepatitis B and C) and he was social alcohol drinking. There was no proof of liver cirrhosis in CT, too. The test results showed HBsAg (-), HBsAb (+), and HCVAb (-) and tumor marker of hepatocellular carcinoma was under the normal range such as Alpha-fetoprotein (α -FP) of 2.2 ng/mL (normal; < 5 ng/mL) and protein induced by vitamin K absence-II of 23 mAU (0-39 mAU). CEA (Carcinoembryonic antigen) and CA 19-9 (carbohydrate antigen) were under the normal range, too. Various other image studies were done. An MRI showed the arterial enhancing lesion, and a PET CT scan showed a hypermetabolic lesion in that same portion of the liver (Figures 3 and 4). We tried to perform a pathological confirmation of the lesion through a liver biopsy. However, we could not obtain the tissue because no typical mass was detected on ultrasonography.

In summary, he had a no risk of hepatocellular carcinoma and normal range of tumor markers, and there was no mass in liver. He was initially far advanced stage of gastric cancer, and he was high risk subtype of recurrence. Base on the data, we assumed it might be an arterial-portal shunt in the liver. However, this phenomenon strongly indicated a recurrent mass in the liver despite of no obvious proof of recurrence such as typical mass or elevated tumor marker (CEA, CA 19-9). After reviewing literature, we found a few case report of an oxaliplatin-induced liver injury, mimicking a metastatic tumor on an image, although it was a slightly different case (mainly colon cancer and different enhance pattern)^[6-10].

Therefore, we continued with the adjuvant che-

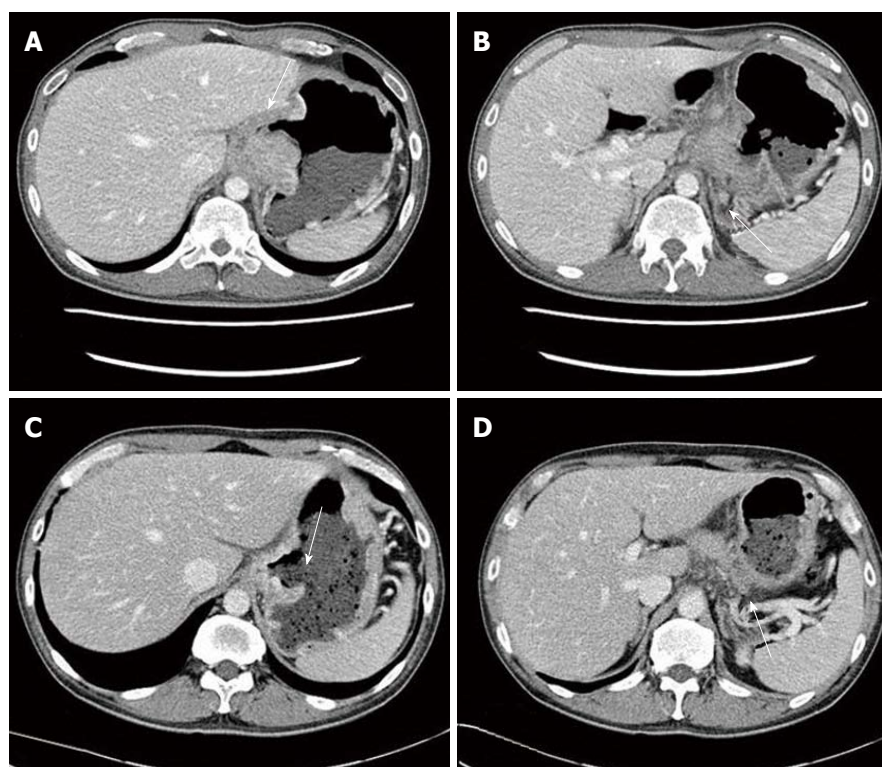


Figure 1 On admission, computed tomography revealed a bulky ulcerative mass with direct invasion of pancreas and left diaphragm (A and B). After 3 cycle of neoadjuvant chemotherapy (capecitabine and oxaliplatin), the mass had markedly decrease (C and D).

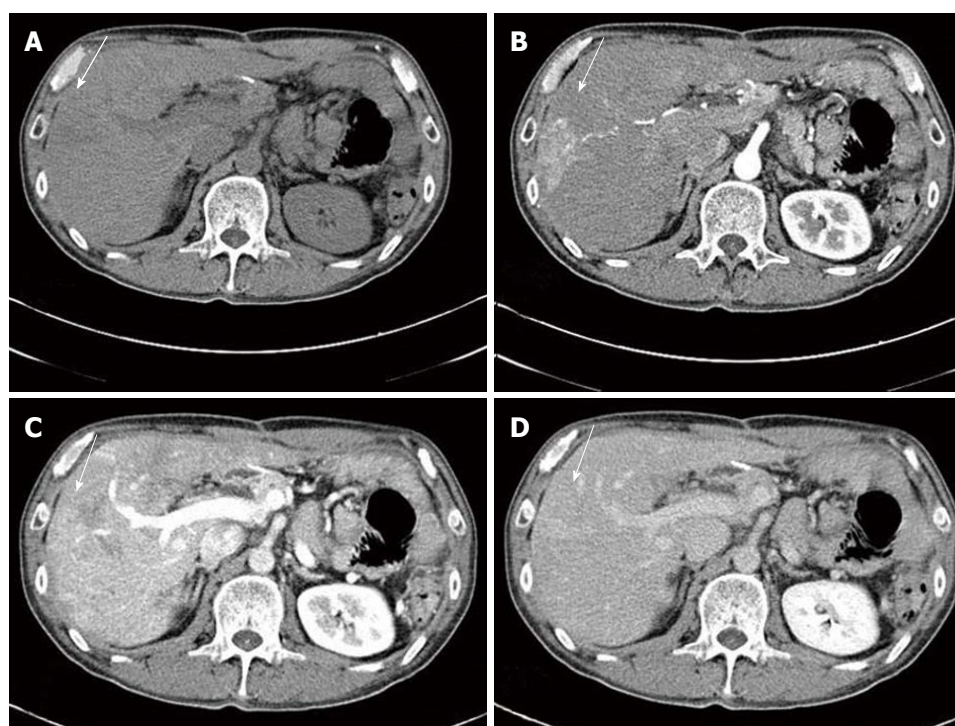


Figure 2 After total six cycle of chemotherapy (capecitabine and oxaliplatin), dynamic computed tomography showed an arterial enhanced mass like lesion. A: Pre-enhanced image; B: Arterial phase image; C: Portal phase image; D: Delayed phase image.

motherapy, this time, eliminating oxaliplatin. After 2 cycles of capecitabine monotherapy, the arterial enhancing mass in the liver disappeared (Figure 5). We

were able to confirm the diagnosis of an AV shunt due to oxaliplatin-induced sinusoidal injury, the patient has not relapsed in 2 years.

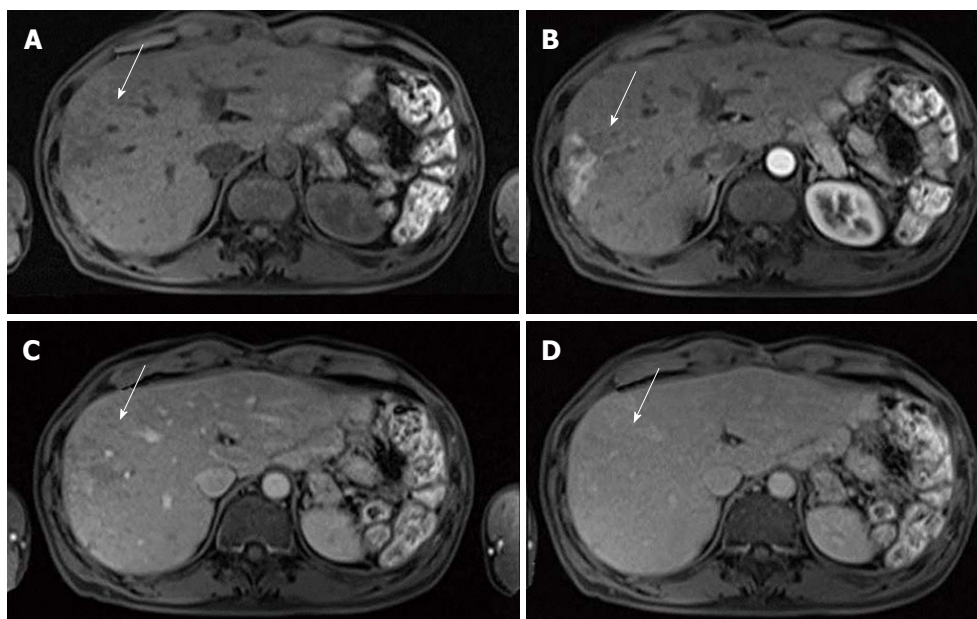


Figure 3 Dynamic magnetic resonance imaging demonstrated an arterial enhance mass like lesion as same as computed tomography scan. A: Pre-enhanced image; B: Arterial phase image; C: Portal phase image; D: delayed phase image.

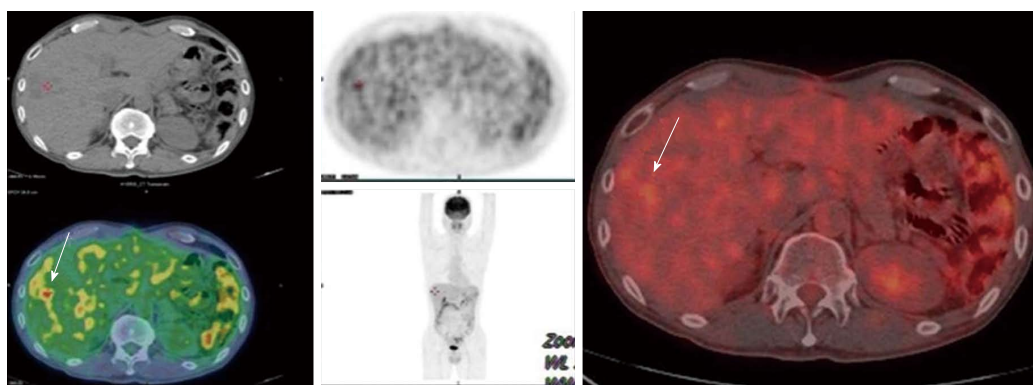


Figure 4 This arterial enhances mass like lesion had a hypermetabolism in positron emission tomography computed tomography.

DISCUSSION

A unique feature of the liver's blood supply system is its dual blood supply, which comes from the hepatic artery (25%) and the portal vein (75%). A perfusion gradient between the artery and portal vein under various conditions such as diminished blood flow through the portal vein can result in an increased attenuation or signal intensity difference during the early phases of a contrast-enhanced dynamic imaging of the liver. In most cases, this finding reflects an increased arterial blood flow, related to an APS. APSs tend to show an amorphous or nodular appearance in the peripheral portion of the liver. There are various causes of APS: hepatic neoplasms, hepatic trauma including interventional procedure, liver cirrhosis, inflammatory diseases, obstruction of the portal or hepatic vein due to various causes, external compression such

as hepatic tumor (hepatocellular carcinoma and cholangiocarcinoma, metastatic tumor, hematoma), or an internal obstruction (tumor thrombus, invasion of cancer). There are various routes, depending on each cause. These routes are transtumoral (through the tumor itself), transversal (due to portal vein tumor thrombosis), transplexal (peribiliary vascular plexus) and transsinusoidal (between microscopic hepatic arterioles and portal venules, distal to portal vein compression or thrombosis), each of which can occur alone or in combination with each other^[2-5].

However, our case was an incidental arterioportal shunt, mimicking a recurrent gastric cancer, on various images (dynamic CT, MRI and PET). Actually, we are uncertain about why the arterial enhancing nodule (suspected APS) spontaneously disappeared. However, our patient had no history of liver cirrhosis, intervention of liver, or liver trauma (including trauma

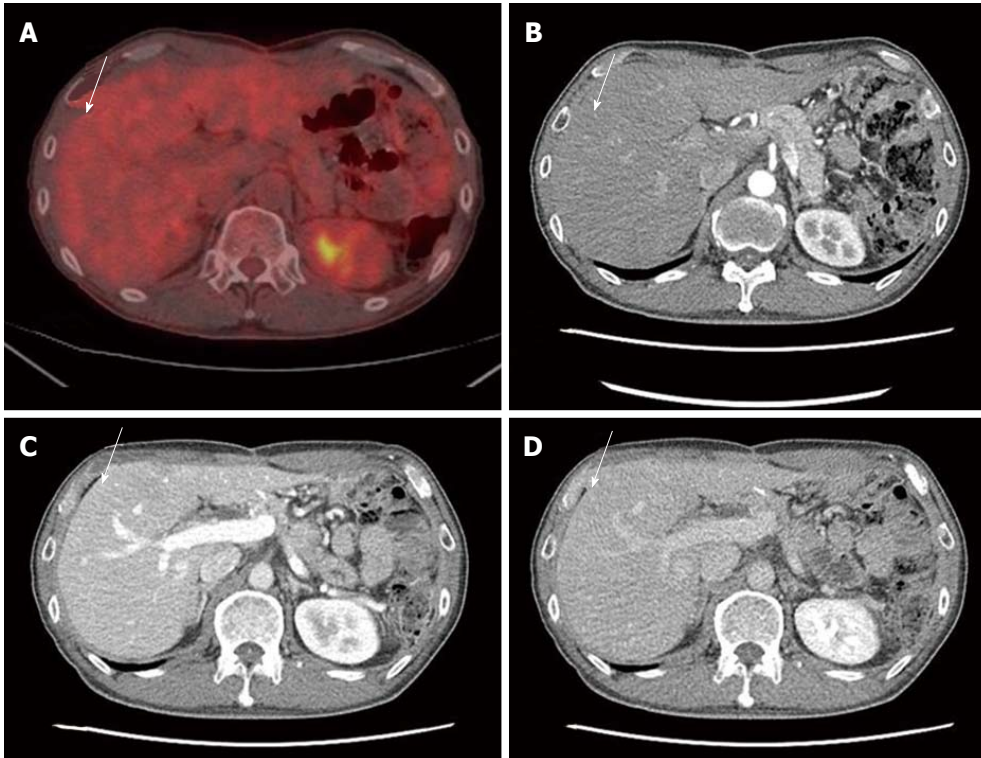


Figure 5 After adjuvant chemotherapy without oxaliplatin, the arterial enhance lesion disappeared. A: PET CT; B: Arterial phase image; C: Portal phase image; D: Delayed phase image.

from surgery of the liver). Moreover, no typical mass was picked up by the sonography.

Oxaliplatin is platinum-based, and a third generation chemotherapeutic agent, administered to patients with gastric cancer, and colorectal cancer. It has comparably less toxic side effects than that of cisplatin. However, there are increasing numbers of reports indicating that oxaliplatin-based chemotherapy could cause damage to a non-tumor-bearing liver. Several reports have confirmed the association between hepatic sinusoidal injury and oxaliplatin. Oxaliplatin-induced hepatic sinusoidal injury can range from a mild sinusoidal dilatation to a hepatic sinusoidal obstruction syndrome. Clinical manifestations vary from asymptomatic to hepatomegaly, jaundice, and ascites. The oxaliplatin sinusoidal injury lesions appeared after the administration of oxaliplatin; however, most lesions disappeared after the cessation of oxaliplatin treatment^[6-10].

The incidence of hepatic sinusoidal injury ranges between 19% and 54%. A retrospective study demonstrated that approximately half the patients who received preoperative chemotherapy with oxaliplatin developed some degree of hepatic sinusoidal dilation and microscopic hemorrhage related to damage to the hepatic sinusoidal endothelial cell barrier^[11,12].

Oxaliplatin-induced liver damage is characterized histologically by sinusoidal dilatation and congestion

outlined by atrophic hepatocyte and/or fibrosis and venular obstruction. An immunohistochemical study of cluster of differentiation 34 revealed a decrease in sinusoidal endothelial cells in this lesion. Such an injury often increases the porosity of the sinusoidal endothelium, and increases cellular fenestrations. This led to the obstruction of the sinusoids and interruption of the portal circulation, resulting in hepatic congestion and eventually elevated portal pressures. Finally, some advanced cases of oxaliplatin-induced sinusoidal injury, have induced veno-occlusive disease, sinusoidal obstruction and portal hypertension^[13-15]. Among the pathogenesis of oxaliplatin-induced sinusoidal injury, hepatic congestion due to an obstruction of the sinusoids and an interruption of portal circulation is similar to APS, through a transsinusoidal route. It is thought that oxaliplatin-induced sinusoidal obliteration of peripheral hepatic venules could lead to a retrograde filling of small portal vein branches, by way of a transsinusoidal route^[2-5].

Our patient underwent splenectomy, and there might be a question that it may affect to oxaliplatin induced sinusoidal injury. However, we couldn't find the relationship between splenectomy and oxaliplatin-induced sinusoidal injury or APS, and splenectomy was rather as option of treatment of hypersplenism from oxaliplatin-induced sinusoidal injury^[16].

In conclusion, although we had no established

evidence of a relationship between oxaliplatin and incidental APS, we concluded on the diagnosis because of the following reasons: Firstly, there was no reason lead to be APS such as trauma, intervention and liver cirrhosis. Secondly, the arterial enhancing lesion appeared after the administration of oxaliplatin and disappeared after the cessation of oxaliplatin. Thirdly, although no definitive mechanism exists for our case, oxaliplatin-induced sinusoidal injury has been identified, and this is enough evidence from literature, to support our case. To the best of our knowledge, this is the first case report of arterioportal shunt incidental to treatment with oxaliplatin that mimics recurrent gastric cancer upon interpretation of multiple imaging modalities.

COMMENTS

Case characteristics

After adjuvant chemotherapy with capecitabine and oxaliplatin in stage IIIb gastric cancer, a computed tomography revealed a 1 cm sized arterial-enhancing nodule in the liver.

Clinical diagnosis

The authors presumed initially a new hepatocellular carcinoma or recurrent gastric cancer.

Differential diagnosis

Hepatocellular carcinoma, recurrent gastric cancer, arterioportal shunt.

Laboratory diagnosis

Various tumor marker were under the normal range.

Imaging diagnosis

Various imaging including dynamic computed tomography (CT), magnetic resonance imaging, and Positron emission tomography-CT showed an arterioportal shunt.

Pathological diagnosis

The authors could not obtain the tissue because no typical mass was detected on ultrasonography.

Treatment

The patient was continued on treatment with adjuvant chemotherapy following discontinuation of oxaliplatin.

Related reports

There was no report of non-tumorous incidental Arterioportal shunt (APS) related to treatment with oxaliplatin.

Term explanation

APS is an organic communication between the hepatic arterial system and the portal venous system.

Experiences and lessons

The authors want to share this case as rare condition of arterioportal shunt due to oxaliplatin induced sinusoidal injury.

Peer-review

This is the first case report of arterioportal shunt incidental to treatment with

oxaliplatin that mimics recurrent gastric cancer upon interpretation of multiple imaging modalities

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Comment on “Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics”

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Abstract

Attaran *et al*^[1] have recently shown that decreased susceptibility of established *Helicobacter pylori* (*H. pylori*) biofilms to specific antibiotics, was associated with the overtly enhanced transcription of two efflux pump genes, *hp1165* and *hefA*, involved in specific resistance to tetracycline and multiple antibiotics, respectively. Apart from antibiotic exposure, secretion of multiple antimicrobial peptides, such as human β -defensins ($h\beta$ DS), by the gastric epithelium upon *Hp* challenge, may act as early triggering events that positively impact biofilm formation and thus, antibiotic resistance. In this regard, we undertook genomic transcriptional studies using *Hp* 26695 strain following exposure to sublethal, similar to those present in the gastric niche, concentrations of $h\beta$ DS in an attempt to provide preliminary data regarding possible mechanisms of immune evasion and selective sensitivity of *Hp*. Our preliminary results indicate that $h\beta$ D exposure ignites a rapid response that is largely due to the activation of several, possibly interconnected transcriptional regulatory networks – origins - that ultimately coordinate cellular processes needed to maintain homeostasis and successful adaptation of the bacterium in the gastric environment. In addition, we have shown that both antibiotic and $h\beta$ D resistance are mediated by dedicated periplasmic transporters, including the aforementioned efflux pump genes *hp1165* and *hefA*, involved in active export of antibiotics from the cell membrane and/or, as recently suggested, substrate sensing and signalling. Furthermore, it

appears that sublethal doses of h β Ds may enhance biofilm formation by the sustained expression of, mainly, quorum sensing-related genes. In conclusion, we provide additional data regarding the role of specific innate immune molecules in antibiotic cross-resistance mechanisms that may deepen our understanding in the context of the development of novel eradication regimens.

Key words: *Helicobacter pylori*; Human β -defensins; Biofilm; Antimicrobial resistance

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Core tip: In the course of *Helicobacter pylori* infection, epithelium-derived human β -defensins may act as early triggering signals that induce biofilm formation and enhanced expression of antibiotic resistance genes, regardless of prior antibiotic exposure.

Kazakos EI, Dorrell N, Polyzos SA, Deretzi G, Kountouras J. Comment on “Effect of biofilm formation by clinical isolates of *Helicobacter pylori* on the efflux-mediated resistance to commonly used antibiotics”. *World J Gastroenterol* 2017; 23(33): 6194-6196 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i33/6194.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i33.6194>

TO THE EDITOR

Attaran *et al*^[1] concluded that, in biofilm-forming populations, overexpression of two efflux pump genes, *hp1165* and *hefA*, conferring resistance to tetracycline and multiple antibiotics respectively, may favor reduced antibiotic susceptibility of *Helicobacter pylori* (*H. pylori*) *in vivo*.

Further to antibiotic exposure, additional, epithelial-derived molecules may function as triggering signals during the dynamic *H. pylori* interaction with the gastric mucosa, provoking overexpression of efflux pumps that in turn, regulate the bacterium's biofilm-producing capacity and promote its virulence. Several studies have unraveled the role of constitutive and/or induced expression of human β -defensins (h β Ds) 1 - 4 in the bacterium's adaptation in the human stomach and *H. pylori* -related pathologies^[2,3].

In this respect, we performed whole genome transcriptome analyses (competitive genomic RNA/RNA hybridisations) using *H. pylori* -specific microarrays based on the *Hp* 26695 and J99 genome sequences and annotation available at the time. Briefly, *H. pylori* 26695 strain was exposed to sublethal, similar to those encountered at the gastric epithelium concentrations of h β Ds, in an attempt to identify possible mechanisms of *H. pylori* immune escape and clarify their role in biofilm development *in vitro*. Our preliminary results have identified profound changes in the transcriptional profile

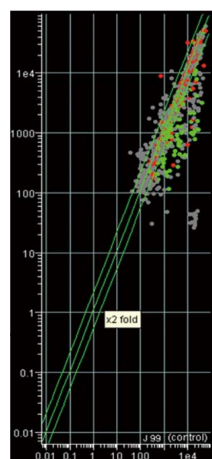


Figure 1 Representative scatter analysis of the general patterns of *H. pylori* genomic response to human β -defensin 3 (h β D3) revealed by transcriptional profiling. Scatter plots (log₂ ratio) of average normalized intensities representing Cy5-red channel versus Cy3-green channel are shown for experiments in the presence of sublethal concentrations of h β D3 compared with “control” conditions (no h β D3). Differential expression of a given gene is reflected by deviation from the central diagonal line. The upper diagonal defines ≥ 2 -fold up-regulation and the lower one defines ≥ 2 -fold down-regulation.

of *H. pylori* 26695 demonstrated by the induction or suppression of multiple gene components of distinct regulatory and signaling cascades activated as a result of environmental stress (Figure 1, unpublished data). Overall, the vast majority of genes affected, encoded components of the cell wall stimulon, possibly as means to prevent h β D-specific binding and proper immune recognition, or could be further assigned to certain origins, essential for colonisation of the gastric niche and long-term adaptation, intracellular metal homeostasis and urease activation that largely determine *H. pylori* pathogenicity. Apart from the marked induction of *hp1165* and *hefA*, also reported by the authors^[1], several other genes coding for transmembrane ABC transporters (*glnP*, *dppF*, *hp1458*, *hp1486*), efflux proteins (*hp0656*, *hp0946*), multidrug and toxic extrusion proteins were found to be significantly up-regulated, thereby indicating their prominent role in the cellular response to h β Ds challenge, membrane detoxification and maintenance of osmotic balance.

Interestingly, enhanced biofilm production by *Hp* 26695, observed in our studies upon exposure to sublethal concentrations of h β D1 and h β D3, was primarily attributed to the down-regulation of *metK* and *luxS* genes, involved in synthesis of quorum-sensing autoinducer-2, in accordance to previously published data^[4,5].

Collectively, our results indicate that sublethal doses of epithelial-secreted antimicrobial peptides such as h β Ds, may select co-resistance to antibiotics commonly used in *Hp* eradication therapies and *vice versa*, considering that they provoke the activation of shared, contact-dependent signaling networks, including efflux pumps. Furthermore, it appears that h β Ds may independently act as triggering stimuli

promoting biofilm formation *in vivo* which in turn, accounts, at least partly, for the observed failure of eradication regimens and the establishment of *H. pylori* -related chronic inflammation.

Given the complexity of *H. pylori* -host epithelial crosstalk aforementioned data warrant further investigation to achieve the development of successful anti-biofilm strategies that will ultimately re-enforce our therapeutic options mainly towards eradication of *H. pylori* -related resistance. Furthermore, future research focus on the polymorphic variability of the human genome that directly affects epithelial dynamics of hβDs expression may reveal important correlation patterns between *H. pylori* pathogenesis, including biofilm formation, and individual disease susceptibility.

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