ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2019 May 7; 25(17): 2019-2148

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

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Weekly Volume 25 Number 17 May 7, 2019

CASE REPORT

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World Journal of Gastroenterology

Volume 25 Number 17 May 7, 2019

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NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORS-IN-CHIEF
Subrata Ghosh, Andrzej S Tarnawski

EDITORIAL BOARD MEMBERS
http://www.wjgnet.com/1007-9327/editorialboard.htm

EDITORIAL OFFICE
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PUBLICATION DATE
May 7, 2019

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World J Gastroenterol 2019 May 7; 25(17): 2019-2028

DOI: 10.3748/wjg.v25.i17.2019

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

OPINION REVIEW

Microbial metabolites in non-alcoholic fatty liver disease

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Supported by National Key Research and Development Plan ' Precision Medicine Research' , No. 2017YFSF090203; the National Natural Science Foundation of China, No. 81470840, No. 81873565, and No. 81800510; and Shanghai Sailing Program, No. 18YF1415900.

Conflict-of-interest statement: No potential conflicts of interest.

Manuscript source: Invited manuscript

Received: February 21, 2019
Peer-review started: February 22, 2019
First decision: March 20, 2019
Revised: March 25, 2019

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Abstract

The prevalence of non-alcoholic fatty liver disease (NAFLD) is rising exponentially worldwide. The spectrum of NAFLD includes non-alcoholic fatty liver, non-alcoholic steatohepatitis, liver cirrhosis, and even hepatocellular carcinoma. Evidence shows that microbial metabolites play pivotal roles in the onset and progression of NAFLD. In this review, we discuss how microbe-derived metabolites, such as short-chain fatty acids, endogenous ethanol, bile acids and so forth, contribute to the pathogenesis of NAFLD.

Key words: Microbial metabolites; Non-alcoholic steatohepatitis; Short-chain fatty acids

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a global epidemic metabolic disease lacking effective therapeutic strategies and the internal pathogenesis is still uncertain. Gut microbiota-derived metabolites have attracted much attention for its association with the onset and progression of NAFLD. In this review, we mainly elucidate the diverse roles of microbe-derived metabolites in the development of NAFLD, which is conducive to better understanding the biological functions of microbial metabolites in NAFLD *via* the gut-liver axis and facilitating the excavation of potential therapeutic approaches for NAFLD.

Citation: Zhou D, Fan JG. Microbial metabolites in non-alcoholic fatty liver disease. *World J Gastroenterol* 2019; 25(17): 2019-2028
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2019.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2019

Accepted: March 29, 2019
Article in press: March 30, 2019
Published online: May 7, 2019

P-Reviewer: Milovanovic T, Sitkin S
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic metabolic disorder that is strongly associated with obesity and metabolic syndrome. NAFLD has become the most common chronic liver disease worldwide[1,2], causing a substantial global health burden. Although the exact pathogenesis of NAFLD is uncertain, in addition to the well-known "two-hit" theory or the multiple-parallel-hits hypothesis[3,4], the dysbiosis of the gut microbiota also promotes the development of NAFLD by mediating the processes of energy metabolism, insulin resistance, immunity, and inflammation[5-7].

The gut flora in the intestinal tract exhibits high diversity and distinct differences, and the total number of bacterial cells can reach 10^{14}[8]. The intestinal bacteria mainly belong to the following phyla: Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia, and Fusobacteria; together, Firmicutes and Bacteroidetes account for up to 90% of all bacterial cells in the human intestine. The gut microbiota is deemed a special "organ" in human beings; bacterial genes are approximately 100-fold more abundant than human genes, and they encode more functional genes[8]. A large proportion of bacterial genes and their biological functions are specific, and the metabolic potential related to the capacity for the conversion and degradation of host-derived substances is strong. Therefore, the gut microbiota exhibits a profound capacity to synthesize or produce many metabolites. Recently, increasing evidence has shown that these metabolites play pivotal roles in the interactions between the gut microbiota and the host in various ways, and the gut-liver axis is the main link between the gut and the liver (Figure 1). Naturally, an imbalance in the intestinal microbiome and the related metabolites contributes to the onset and progression of NAFLD[9,10]. The accurate pathological diagnosis of NAFLD relies on a liver biopsy; however, with further investigation, the gut microbiota and its metabolites may serve as potential biomarkers for NAFLD and non-alcoholic steatohepatitis (NASH). A clinical study demonstrated that certain gut microbiome-derived metabolites shared gene-effects with hepatic steatosis and liver fibrosis[11,12]. In addition, another study used targeted metagenomics and metabolomics analysis to demonstrate that a decrease in *Oscillospira* accompanied by upregulation of 2-butanone and an increase in *Ruminococcus* and *Dorea* were signatures of non-alcoholic fatty liver (NAFL) onset and NAFL-NASH progression[13]. However, additional validations with clinical samples are needed.

Recently, several original investigations showed that the severity of NAFLD is associated with changes in the levels of certain metabolites in the serum; although not all such metabolites are synthesized or produced by gut bacteria[12,14-16], a better understanding of the role of these metabolites in the development of NAFLD will be valuable for the discovery of new non-invasive diagnostic and treatment options for NAFLD.

SHORT-CHAIN FATTY ACIDS (SCFAS)

The most important bacterial metabolites are SCFAs, which contain fewer than six carbon atoms and have become an increasingly studied gut metabolite due to their multiple biological functions in the liver[17]. The fermentation of dietary fibers by gut bacteria, including *Roseburia*, *Ruminococcus*, *Salmonella*, *Blautia*, *Eubacterium*, *Anaerostipes*, *Coprococcus*, *Faecalibacterium*, *Marvinbryantia*, and *Megasphaera*, is the main source of SCFAs. The most abundant SCFAs present in the colon lumen are acetate, propionate, and butyrate[18]. SCFAs not only provide energy for the intestinal epithelium, but they also have many bioactive roles, such as the regulation of immunity, lipometabolism, and glycometabolism, and the maintenance of gut microbiota homeostasis. SCFAs are involved in the pathogenesis of NAFLD after their absorption and delivery to the liver *via* the portal vein. A clinical study showed that propionate supplementation significantly reduced weight gain and intrahepatocellular lipid content, prevented deterioration in the case of insulin sensitivity, and significantly stimulated the release of peptide-YY and glucagon-like peptide-1 (GLP-1) from human colonic cells; these hormones are closely related to energy metabolism[19]. Another clinical study showed that the total amount of SCFAs was higher in obese subjects compared with lean subjects and, moreover, the ratio of the phyla *Firmicutes* to *Bacteroidetes* was altered in favor of *Bacteroidetes* in obese humans[20]. Basic studies have shown that butyrate-producing probiotics corrected high-fat diet (HFD)-induced enterohepatic immunologic dissonance and attenuated steatohepatitis in mice, which is mediated in part through SCFAs[21-23]. A clinical study showed that a select group of SCFAs-producing bacterial strains played pivotal roles in regulating glucose and lipid metabolism, in part through increased GLP-1

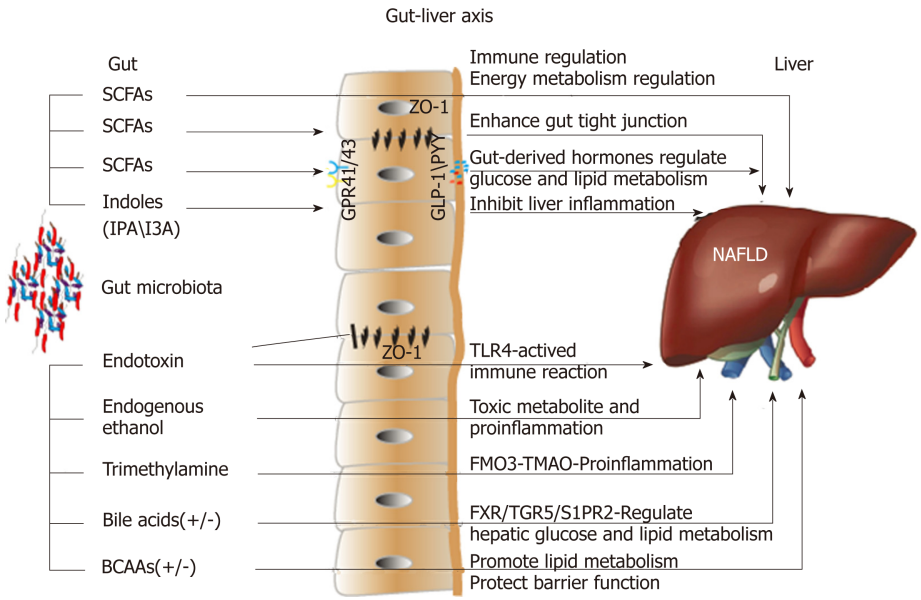

Figure 1 Effects of microbial metabolites on non-alcoholic fatty liver disease *via* the gut-liver axis. SCFAs: Short-chain fatty acids; I3A: Indole-3-acetic acid; IPA: Indole propionic acid; GPR41/43: G-protein-coupled receptors 41/43; ZO-1: Zonula occludens 1; GLP-1: Glucagon-like peptide-1; PYY: Peptide YY; TLR4: Toll-like receptor 4; FMO3: Flavin-containing monooxygenase 3; TMAO: Trimethylamine-N-oxide; FXR: Farnesoid X receptor; TGR5: Takeda G-protein-coupled receptor 5; S1PR2: Sphingosine 1-phosphate receptor 2; BCAAs: Branched-chain amino acids.

production; therefore, the targeted restoration of these SCFA producers may present a novel ecological approach for managing metabolic syndrome and NAFLD[24].

Increasing studies have revealed that SCFAs exert their biological functions mainly *via* activating the G-protein-coupled receptor (GPR) 41/43 or through the inhibition of histone deacetylase (HDAC). Animal experiments showed that GPR41 and GPR43 were involved in lipid and immune regulation, and GPR41/43 deficiency protected against HFD-induced obesity, insulin resistance, and dyslipidemia, in part *via* increased energy expenditure and the promotion of gut-derived hormone GLP-1[25-27]. In addition, the activation of GPR41/43 has been suggested to participate in the pathogenesis of NAFLD. As mentioned above, except for the activation of GPRs, SCFAs can inhibit HDAC directly and regulate the transcriptional activation of genes; among the SCFAs, butyrate is the most powerful HDAC inhibitor[28]. Previous animal studies showed that sodium butyrate supplementation could attenuate HFD-induced NASH, and the underlying mechanisms were associated with restoring the dysbiosis of gut microbiota and improving the gastrointestinal barrier, thereby inhibiting the delivery of gut-derived endotoxin into the liver[29]. Recently, an investigation found that the expression of hepatic GLP-1 receptor was significantly down-regulated in patients with NAFLD, and supplementation with butyrate enhanced hepatic GLP-1R expression in an NASH mouse model by inhibiting histone deacetylase-2 and activating AMP-activated protein kinase (AMPK). These findings indicated that butyrate could be a GLP-1 sensitizer and could prevent the progression of NAFL to NASH *via* promoting the expression of hepatic GLP-1R[30].

In general, SCFAs are thought to be beneficial prebiotics; however, a recent study demonstrated that the soluble fiber inulin, when fermented by gut bacteria into SCFAs, could induce icteric hepatocellular carcinoma (HCC)[31], which was an astounding finding. However, SCFA-induced HCC was shown to be conditional and microbiota-dependent, and this condition was observed in dysbiotic mice; meanwhile, the inhibition of fermentation reduced intestinal SCFAs and prevented HCC. Thus, the enrichment of fermentable fiber should be promoted with caution, and the intake of diverse types of dietary fiber should be emphasized to establish and maintain healthy gut microbiota. This topic still requires further investigation for the design, production, and supply of rational food additives to improve human health. According to various studies, it is very difficult to draw accurate conclusions about the roles of gut microbiota and SCFAs in NAFLD because confounding factors are extensive and cannot be ignored[32].

ENDOGENOUS ETHANOL AND ENDOTOXINS

It is well known that ethanol is a substance that can contribute to hepatic steatosis and inflammation and increase the risk of liver fibrosis and HCC[33]. Endogenous ethanol produced by bacterial fermentation (mainly by *Ruminococcus*) stimulates oxidative stress and aggravates liver inflammation in NAFLD, which was confirmed in animal experiments[34]. Zhu *et al*[35] showed that children and adolescents with NASH harbored more ethanol-producing bacteria in the gut and exhibited higher serum levels of ethanol; moreover, the expression of genes involved in alcohol metabolism was enhanced. Another clinical study showed that children with NAFLD had significantly higher serum levels of ethanol, which were associated with a greater abundance of *Gammaproteobacteria* and *Prevotella*[36]. However, another study demonstrated that the increased blood ethanol levels in patients with NAFLD might result from insulin-dependent impairments of ethanol dehydrogenase activity in the liver rather than an increase in endogenous ethanol synthesis[37]. Although these studies did not all produce consistent results, endogenous ethanol might play a pivotal role in the pathogenesis of NASH. Future investigations are required to determine the exact influence of endogenous ethanol on NAFLD and NASH.

Systemic low inflammatory state is related to the insulin resistance (IR) which contributes to the onset of NAFLD. Gram-negative bacteria-derived endotoxins such as lipopolysaccharide (LPS) were proved to stimulate and aggravate hepatic necroinflammation. The increased intestinal permeability and dysbiosis of gut microbiota promote the translocation of microbial products from intestinal lumen to the liver *via* the portal vein[38]. Toll-like receptors (TLRs), including TLR4, were involved in the LPS-induced liver damage, and LPS could activate TLR4-myeloid differentiation primary-response gene 88 (Myd88) signaling pathway, causing IR, hepatic steatosis, liver inflammation, and fibrosis[39,40]. In addition, Kupffer cells positive for cluster of differentiation 14 (CD14) could enhance LPS-TLR4 response in the liver[41].

BRANCHED-CHAIN AMINO ACIDS (BCAAS)

BCAAs are produced by proteolytic fermentation in the colon. Species implicated in proteolytic fermentation include *Clostridium*, *Fusobacterium*, *Bacteroides*, *Actinomyces*, *Propionibacterium*, and *Peptostreptococci*[42]. Patients with NAFLD have dysregulation of BCAA metabolism, the BCAAs including leucine, valine, and isoleucine were higher both in the blood and urine samples from NAFLD patients[16], the circulating BCAAs were negatively correlated with hepatic insulin sensitivity, and the baseline valine level was identified to be predictive of liver fat accumulation[43]. Meanwhile, BCAAs could reflect hepatic steatosis independently of routine metabolic risk factors, and the metabolic aberrations of BCAAs may precede the development of NAFLD to a certain extent[44]. The increased BCAA levels (valine, leucine, and isoleucine) and downstream BCAA metabolites, such as branched-chain keto acids and short-chain acylcarnitines, were associated with a greater body mass index (BMI)[45]. Further animal experiments showed that BCAA supplementation reduced HFD-induced overweight, but caused obvious liver damage in HFD mice, which was associated with the abnormal lipolysis[46]. Oppositely, several studies indicated that BCAA intervention could alleviate NASH in animal models *via* inhibiting triglyceride deposition in hepatocytes and reducing oxidative and endoplasmic reticulum stress[47-50]. BCAAs were found to have the ability to improve immune function, decrease susceptibility to pathogens, promote the growth of intestinal beneficial bacteria, and enhance the intestinal barrier function[51], all of which appear to prevent the gut-derived toxic substances into the liver.

According to the inconsistent results, limited information is available about the accurate role of BCAAs in the metabolic diseases; it may be valuable to develop diagnostic biomarkers for NAFLD. Further research examining proteolytic fermentation may be vital to understand the interaction between the BCAAs and NAFLD.

BILE ACIDS

Bile acids are not directly produced by the intestinal microbiota; rather, they are mainly synthesized in the liver by using cholesterol as the substrate. Bile acids can be deconjugated and dehydroxylated by the gut microbiota, and the enterohepatic circulation of bile acids, which are reabsorbed and returned to the liver *via* the portal vein, perform many biological functions involved in lipid and glucose metabolism, and are linked to the pathogenesis and treatment of NASH. In an early clinical trial

using Danning Pian (a traditional Chinese medicine that regulates bile acid metabolism) to treat NAFLD, clinical symptoms, serum alanine transaminase levels, blood lipid profiles, and ultrasound-based fatty liver were significantly improved after three months of treatment[52]. Studies show that patients with NASH exhibit alterations in their bile acid profile. The serum levels of bile acids were shown to be elevated in patients with NASH, including the more hydrophobic and cytotoxic secondary species; this increased bile acid exposure may be involved in the pathogenesis of NAFLD[53]. Furthermore, another clinical study demonstrated that increased bile acids were significantly associated with higher grades of heaptic steatosis (taurocholate), lobular (glycocholate) and portal inflammation (taurolithocholate), and hepatocyte ballooning (taurocholate), while the conjugated cholate and taurocholate directly and secondary to primary bile acid ratio was inversely correlated to NAFLD activity score[54]. These results indicated a relationship between the specific bile acids and the histological features of NASH.

Obesity is strongly associated with NAFLD and HCC[55]. Recently, deoxycholicacid (DCA) and the senescence-associated secretory phenotype (SASP) axis were found to have crucial roles in promoting obesity-associated HCC in mice; obesity induces alterations in the gut microbiota and contributes to the increase in DCA, which can cause DNA damage. Moreover, the enterohepatic circulation of DCA promotes the SASP in hepatic stellate cells, which consequently secrete various inflammatory and tumor-promoting factors in the liver. Hence, HCC development was exacerbated in mice after exposure to this chemical carcinogen[56]. This work inferred that maintaining a balanced intestinal microbiota should be advocated, and weight loss may be an effective method.

In addition, bile acids are ligands for the nuclear receptor farnesoid X receptor (FXR). FXR-mediated signaling has beneficial effects on hepatic lipid and carbohydrate metabolism, and this signaling pathway also modulates primary bile acid synthesis in the liver. A previous study found that the serum concentration of bile acids was increased in patients with NAFLD, and the FXR antagonistic deoxycholic acid was also increased, whereas the agonistic chenodeoxycholic acid and the serum level of fibroblast growth factor 19 (FGF 19) were decreased in NAFLD; these alterations contribute to the suppression of hepatic FXR-mediated and fibroblast growth factor receptor 4-mediated signaling, thereby exacerbating NAFLD[57]. This study indicated that targeting FXR signaling might be helpful to the intervention of NAFLD. Fan *et al*. reported that ursodeoxycholic acid (UDCA) combined with a low-calorie diet had therapeutic effects on steatohepatitis in rats[58]. A clinical study demonstrated that patients with NASH who were treated with obeticholic acid (an activator of FXR) exhibited improvements in liver fibrosis, hepatocellular ballooning, steatosis, and lobular inflammation, although the long-term benefits and safety of this drug treatment require further clarification[59]. Newer synthetic FXR agonists that are currently being investigated might cause fewer side effects and exert more powerful effects against NASH[60]. Except for FXR, bile acids are ligands for the cell membrane G-protein-coupled bile acid receptor 1 (known as Takeda G-protein-coupled receptor 5 [TGR5]). TGR5 can regulate inflammation and glucose homeostasis in the liver, which may be associated with the release of GLP-1 and the inhibition of the NLRP3 inflammasome; meanwhile, the activation of TGR5 results in sustained weight loss, improved hepatic steatosis, remitted insulin resistance, and increased energy expenditure in mice[61,62]. Nagahashi *et al*[63] found that the conjugated bile acids can activate the ERK1/2 and AKT signaling pathways *via* sphingosine 1-phosphate receptor 2 (S1PR2) in rodent hepatocytes and *in vivo* to regulate hepatic lipid metabolism. Overall, bile acids are important substances for communication between the liver and the gut; therefore, therapeutically targeting bile acid-related pathways warrants further exploration.

TRIMETHYLAMINE

The nutrient choline was first classified as an essential nutrient due to its physiological function in the prevention of NAFLD[64]. Choline deficiency can lead to NAFLD; thus, a choline-deficient diet is widely used in animal models of NASH[65]. Choline is mainly obtained from the diet, and studies have shown that choline was metabolized to trimethylamine (TMA) by the gut microbiota including *Proteus penneri*, *Escherichia fergusonii*, *Proteus mirabilis*, and other bacteria which can cut the C-N bond of choline[66,67]. TMA is absorbed into the liver *via* the portal vein and oxidized by hepatic flavin-containing monooxygenases into trimethylamine-N-oxide (TMAO)[68]. TMAO is found to contribute to many metabolic diseases, such as cardiovascular diseases, type 2 diabetes mellitus, and NAFLD. A clinical study found that the

circulating levels of TMAO were inversely associated with the severity of NAFLD; in particular, the serum levels of TMAO, choline, and the betaine/choline ratio were shown to be adversely associated with the scores of steatosis and total NAFLD activity. Moreover, the severity of NAFLD was independently correlated with higher serum levels of TMAO, lower levels of betaine, and a lower ratio of betaine/choline[69]. Although the direct mechanisms through which TMA is involved in the onset and progression of NAFLD require further investigation, another clinical study demonstrated that the serum levels of TMAO increased along with BMI and were strongly associated with the fatty liver index, suggesting that a specific cut-off value of serum TMAO might help to identify subjects who are at high risk for NAFLD[70]. Animal experiments showed that in HFD-fed 129S6 mice, the impaired glucose homeostasis and NAFLD occurred, which were associated with disruptions in choline metabolism; meanwhile, the circulating plasma levels of phosphatidylcholine were lower, and the urinary excretion of methylamines was higher, indicating the crucial role of the metabolic balance of choline by the gut microbiota[71]. In addition, previous experiments demonstrated that supplementation with TMAO along with an HFD exacerbated impaired glucose tolerance, obstructed the hepatic insulin signaling pathway, and caused adipose tissue inflammation in mice[72]. Moreover, blocking the TMAO-producing enzyme flavin-containing monooxygenase 3 (FMO3) can regulate obesity and the beiging of white adipose tissue[73]. There are also inconsistent results showing that supplementation with TMAO in HFD-fed mice attenuated impaired glucose tolerance and increased insulin secretion. Therefore, the effects of TMAO on NAFLD might be a double-edged sword[74]. In addition, TMAO can influence cholesterol transport, thereby reducing the synthesis of bile acids and decreasing the production of very low-density lipoprotein (VLDL)[75-77]. In research on other disease, the inhibition of TMA production exhibited beneficial effects on cardiometabolic diseases[67]. Therefore, further well-designed studies are needed to explore the effects of TMA on metabolic syndrome and NAFLD.

TRYPTOPHAN METABOLITES

In addition to the aforementioned gut microbiota-derived metabolites, tryptophan metabolites have been shown to affect the development of NAFLD. Indoles are the main tryptophan-derived gut bacterial products, which include indole-3-acetic acid (I3A), indole propionic acid (IPA), indole-3-lactic acid, indole-3-carboxylic acid, and tryptamine, mainly produced by *Bacteroides*, *Eubacterium*, and *Clostridium*[18]. I3A and tryptamine reduced the production of pro-inflammatory cytokines by macrophages and inhibited macrophage migration to monocyte chemoattractant protein-1. In addition, I3A could alleviate cytokine-mediated lipogenesis in hepatocytes *via* the activation of the aryl-hydrocarbon receptor[78]. This study suggests that I3A and tryptamine are crucial metabolites that mediate host-microbiota crosstalk. Further studies are warranted, including animal experiments and clinical investigations, to determine whether I3A and tryptamine can effectively alleviate NAFLD.

Obesity is definitely associated with the morbidity of NAFLD, and supplement of IPA obviously reduced weight gain in animal experiments[79]. Previous work showed that IPA could scavenge free radicals and reduce oxidative stress[79], and IPA was thought to be a candidate for treatment of metabolic disorders as for its beneficial effects on glucose metabolism and insulin resistance[80]. Besides, IPA was found to be lower in obese subjects, and the elevation of plasma IPA level improved intestinal barrier function *in vitro* and *in vivo* through the combination of IPA and pregnane X receptor[81,82], which in turn inhibited the endotoxin-induced TLR4 signaling and improved tissue inflammation. Taken all together, further basic and clinical research on the tryptophan-derived microbial metabolite may be crucial for understanding their implications in obesity and NAFLD.

CONCLUSION

NAFLD has become the most common chronic liver disease worldwide. The interactions between the gut microbiota and NAFLD have been widely investigated, and advances in understanding the molecular mechanisms underlying the gut-liver interactions are critical to the development of non-invasive serum biomarkers and targeted therapies for NAFLD and NASH. To date, the precise association between the gut microbiota and NAFLD, as well as an accurate definition of a healthy gut microbiota, are still difficult to conclude; however, the gut microbiota is undoubtedly a contributing pathogenic factor in NAFLD, and the microbial metabolites serve as a

key bridge between the gut microbiota and NAFLD *via* the gut-liver axis.

Although alterations in microbial metabolites may be remarkable therapeutic targets or excellent biomarkers for NAFLD, conclusions from different studies are inconsistent due to numerous uncontrollable factors that influence the results. Therefore, unified research standards, detection methods and conditions, and evaluation approaches should be established. On the other hand, translational and precision studies including a single species or a specific bacterial group, and a single signaling pathway molecule associated with bacterial metabolism should be employed for improvements of human health. In addition, a larger and more comprehensive clinical cohort from the world is indispensable. Different races, environments, and genetic backgrounds should be effectively distinguished, which would help to obtain more accurate, stable, and applicable results. Hopefully, individualized treatment for NAFLD targeting the gut microbiota or microbial metabolites will be revealed in the near future.

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World Journal of Gastroenterology

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World J Gastroenterol 2019 May 7; 25(17): 2029-2044

DOI: 10.3748/wjg.v25.i17.2029

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Recent advances in gastric cancer early diagnosis

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Author contributions: All authors equally contributed to this paper with conception and design of the study, literature review and analysis, drafting and critical revision and editing, and final approval of the final version.

Supported by a grant of the Romanian National Authority for Scientific Research and Innovation, CNCS - UEFISCDI, No. PN-III-P4-ID-PCCF2016-0158 (contract PCCF 17/2018), within PNCDI III.

Conflict-of-interest statement: No potential conflicts of interest.

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Abstract

Gastric cancer (GC) remains an important cause of cancer death worldwide with a high mortality rate due to the fact that the majority of GC cases are diagnosed at an advanced stage when the prognosis is poor and the treatment options are limited. Unfortunately, the existing circulating biomarkers for GC diagnosis and prognosis display low sensitivity and specificity and the GC diagnosis is based only on the invasive procedures such as upper digestive endoscopy. There is a huge need for less invasive or non-invasive tests but also highly specific biomarkers in case of GC. Body fluids such as peripheral blood, urine or saliva, stomach wash/gastric juice could be a source of specific biomarkers, providing important data for screening and diagnosis in GC. This review summarized the recently discovered circulating molecules such as microRNAs, long non-coding RNAs, circular RNAs, which hold the promise to develop new strategies for early diagnosis of GC.

Key words: Biomarkers; Gastric cancer; Early diagnosis; Genetic and epigenetic alterations; Circulating molecules

Core tip: Despite the fact that in the last decades, gastric cancer (GC) has shown a decreasing incidence, the five-year survival rate continues to remain poor mainly because most patients are asymptomatic until the disease progresses to advanced stages. Recent progress in molecular landscape of GC and improved detection methods may facilitate screening and diagnosis of GC in early stages. Numerous studies aim to identify specific non-invasive biomarkers from alternative sources such as peripheral blood, urine or saliva, stomach wash/gastric juice. This review summarized the recently

Manuscript source: Invited manuscript

Received: March 19, 2019
Peer-review started: March 19, 2019
First decision: March 27, 2019
Revised: April 3, 2019
Accepted: April 19, 2019
Article in press: April 20, 2019
Published online: May 7, 2019

P-Reviewer: Aoyagi K, Chiu CC, Kim BW, Tanabe S
S-Editor: Ma RY
L-Editor: A
E-Editor: MaYJ

discovered circulating molecules which hold the promise to develop new strategies for early diagnosis of GC.

Citation: Necula L, Matei L, Dragu D, Neagu AI, Mambet C, Nedeianu S, Bleotu C, Diaconu CC, Chivu-Economescu M. Recent advances in gastric cancer early diagnosis. *World J Gastroenterol* 2019; 25(17): 2029-2044
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2029.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2029

INTRODUCTION

Gastric cancer (GC) remains a challenge for oncology domain being the fifth most frequently diagnosed cancer (1033701 new cases in 2018) and the third leading cause of cancer death (782685 deaths) of all malignancies worldwide[1]. Although over the last decades GC has shown a decreasing incidence, the five-year survival rate continues to be poor, being estimated at 10% for patients with advanced GC. In the developed countries, like Japan, where early diagnosis of GC reaches 50%, the five-year survival rate attains 90%[2].

Currently, the most frequent tumor markers used in the clinic for early detection of GC comprise carcinoembryonic antigen (CEA), the carbohydrate antigens (CA) - CA19-9, CA72-4, CA125, CA24-2, CA50, and also pepsinogen and α-fetoprotein (AFP)[3]. However, the specificity and sensitivity of these serum biomarkers are poor and so far, none of them is unique for GC diagnosis[3,4]. Thereby, the development of improved detection method to diagnose CG in early stages is crucial, especially knowing that most patients are asymptomatic until the disease progresses to advanced stages. Moreover, GC is a complex, heterogeneous disease, involving multiple genetic and epigenetic alterations[5].

Recently, the use of high throughput technologies has brought new insights into the molecular pathogenesis, resulting in a new molecular classification of gastric adenocarcinoma into four subtypes, based on their genomic features. According to The Cancer Genome Atlas (TCGA), GCs are divided in Epstein-Barr virus (EBV)-infected tumors, microsatellite instability tumors (MSI), genomically stable tumors (GS), and chromosomally unstable tumors (CIN)[6]. The Asian Cancer Research Group (ACRG) categories GC into MSI tumors and Microsatellite Stable (MSS) tumors with either epithelial-to-mesenchymal transition (MSS/EMT), TP53 activity (MSS/TP53+), or TP53 inactivity (MSS/TP53-)[7,8]. This new classification opened the way for several clinical trials that are trying to define new therapeutic regimens combining immune checkpoint inhibitors with molecular targeted therapies, with promising results[9]. However, early diagnosis remains mandatory, and studies aiming to identify new biomarkers or genetic signatures are imperative.

Genetic alterations, including large chromosomal gain or loss, single nucleotide variations, and mutations, as well as epigenetic alterations, like aberrant DNA methylation, histone modification, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) overexpression or down-regulation, were described as major aspects implicated in GC initiation and progression[10].

A better understanding of the molecular factors involved in gastric carcinogenesis can lead to the identification of novel biomarkers for early GC diagnosis or markers use for prognosis and for monitoring therapy response. This review aims to discuss the most important types of molecules secreted from the tumor tissues to the body fluids, which candidates as circulating biomarkers for early diagnosis of GC (Figure 1).

CIRCULATING PROTEOMIC BIOMARKERS IN EARLY GC

Although several circulating tumor-associated antigens have entered routine clinical practice for a long time their utility in early detection of GC remains elusive, due to the high incidence of false-positive and false-negative results[11,12]. CEA, CA19-9, and CA72-4 are the most frequently used conventional tumor markers in GC diagnosis, prognosis, therapeutic monitoring and detection of recurrences[13]. At diagnosis, both CEA and CA 19-9 levels can provide useful prognostic information regarding the depth of tumor invasion and the presence of metastases[14,15]. However, they do not

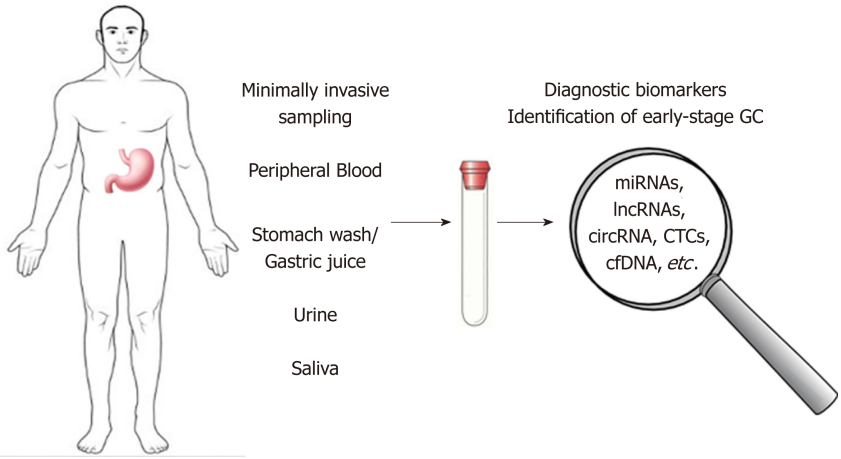

Figure 1 Possible non-invasive diagnostic biomarkers for early-stage gastric cancer. Genetic and epigenetic alterations, microRNAs, long non-coding RNAs and circular RNA, circulating tumor cells and tumor DNA represent promising candidates for the development of new non-invasive methods in early-diagnosis of gastric cancer. GC: Gastric cancer; miRNAs: MicroRNAs; lncRNAs: Long non-coding RNAs; circRNA: circular RNA; CTCs: Circulating tumor cells; cfDNAs: Cell-free circulating DNA.

represent effective tools for GC screening and early diagnosis as they do not display enough sensitivity and specificity under these circumstances[16,17]. CA72-4 was shown to exhibit higher sensitivity and accuracy than CEA, yet there are only few studies that investigated its relevance in GC screening[18]. Other tumor markers, such as AFP and CA125 proved to have very low positivity rates in early GC[19]. Also, CA50 is of limited diagnostic value[20].

To increase the diagnostic performance for GC different combinations of serological tumor markers were employed. In this respect, it was shown that by combining CEA, CA19-9, and CA72-4 with thymidine kinase 1 (TK1) - a biomarker of cell proliferation - a significant increase in sensitivity and specificity of GC detection was obtained, compared to the isolated use of the biomarkers[21]. Recently, a diagnostic model including the serum levels of CEA, CA72-4 and of three inflammatory cytokines [tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8] was proposed for early GC detection. In the validation study, this model provided good discrimination between healthy controls, atypical hyperplasia of gastric mucosa, early-stage GC and advanced-stage GC groups[22].

Concerning the use of stomach-specific biomarkers, measurement of serum pepsinogens (PGs) is the most common non-invasive method employed for GC detection, although it identifies individuals with gastric precancerous lesions, rather than GC itself[23]. Thus, low levels of pepsinogen I (PGI) and a low pepsinogen-I to pepsinogen ratio (PGI/PGII) exhibit a good correlation with atrophic changes in the gastric corpus, while their accuracy for GC detection is low[24]. Additionally, gastrin-17 (G-17) was proposed as an indicator antral atrophy[25]. As shown recently, a biomarker panel comprising PGI, PGII, PGI/PGII, G-17 and IgG antibodies to *Helicobacter pylori* (*H. pylori*) represents a promising non-invasive tool to stratify individuals at high risk for GC development[26]. Also, serum levels of trefoil factor 3 (TFF3), a protein ectopically expressed in intestinal metaplasia of the stomach[27], was found to display better performance in GC detection than PGs[25], and the combination of TFF3 with PGs demonstrated even higher sensitivity for early GC[28].

Other potential circulating biomarkers for detecting early-stage GC include M2-pyruvate kinase, a tumor-associated metabolic marker; the adipocytokine leptin as an independent biomarker of intestinal metaplasia; p-53 autoantibody; the cell-cycle-related protein RegIV; the inflammatory signaling molecules olfactomedin 4 and vascular adhesion protein-1 (VAP-1)[29].

A different approach to identify early GC biomarkers has involved mass spectrometry for analyzing serological glycomic profiles in GC patients and non-cancer controls. Significant differences in serum N-glycans were observed between the two groups. Moreover, the decreased core fucose was validated as a potential biomarker for distinguishing early-stage GC patients from healthy controls[30].

ONCOGENES/TUMOR SUPPRESSORS IN GC

The development of state-of-the-art techniques holds the promise of new molecular markers identification that are able to diagnose early, predict the disease outcome and help access the appropriate therapy. Numerous studies showed an increased level of expression of oncogenes in GC. They stimulate tumor cell growth and cell cycle and inhibit apoptosis.

Recent studies identified several genes whose elevated expression level proved to be associated with GC and might be useful in early detection, such as *xpg*, interferon-induced transmembrane protein 1 (*iftim1*), matrix metalloproteinase-9 (*mmp-9*), pituitary tumor-transforming gene-1 (*pttg1*), *stc1*[31].

XPG/ERCC5 (13q33) Xeroderma pigmentosum group G/excision repair cross-complementing group 5, an enzyme from NER (nucleotide excision repair) system, is involved in repairing of DNA lesions caused by genomic instability. The gene expression level of *ercc5* was found to be significantly higher in GC compared to gastritis, and it was associated with tumor development and progression[32].

By microarray profiling methods, *ifitm1* was identified as a gene upregulated in tumor cell lines and GC tissues. Moreover, important differences in expression level in intestinal vs diffuse type of GC were observed. Although the role of this gene in tumorigenesis is not clearly understood, *ifitm1* raised expression was implicated in invasion and migration of GC cells[33] and was also related to increased inflammatory responses that may play a part in tumor progression.

MMP-9 is an enzyme that contributes to the degradation of the extracellular matrix, having a well-known role in tumor growth, invasion and metastasis in gastric carcinoma[34]. A study that evaluates both serum and tissue expression level of MMP-9, found out a correlation between serum concentration of MMP-9 before surgery and TNM staging. Although the *mmp-9* expression level in gastric tumor was higher compared to healthy tissue, and positively associated with depth of invasion, it did not correlate significantly with MMP-9 serum level[35].

A recently discovered proto-oncogene, *pttg1* can affect tumorigenesis, invasion, and metastasis of many cancer types. The expression of *pttg1* is upregulated in gastric tumor tissue compared to gastric intraepithelial neoplasia and normal mucosa (both mRNA and protein level) and it is an independent factor for survival. PTTG1 might represent a potential diagnostic marker and a therapeutic target[36].

STC1 and STC2, members of STC (stanniocalcin) family, were highly expressed in numerous cancer types. In GC, both STC1 and STC2 expression is upregulated, STC1 being significantly associated with tumor staging, metastasis, and progression-free survival. Serum level of STC1 was significantly elevated in preoperative cancer patients compared to benign gastric cases and decreased 7-10 d after surgery[37]. Arigami *et al*[38] reported a significantly higher number of *stc1* mRNA copies in the blood of GC patients *vs*. normal controls that correlates with tumor invasion and staging and has a greater sensitivity than CA 19-9 and CEA. These studies suggest the utility of serum STC1 as diagnosis and prognosis marker in GC.

Using gene microarray, our group also identified a panel of overexpressed genes associated with tumor progression: KRT17, COL10A1, KIAA1199, SPP1, IL11, S100A2, and MMP3. From these, COL10A1, KRT17, and SALL4 candidate as biomarkers for early detection having an increased expression in the early stages of gastric tumorigenesis[39]. COL10A1 was found elevated in serum of patients with colorectal cancer[40], proven to be a worthy circulating biomarker for early diagnosis. KRT17 was also demonstrated to be involved in tumor growth, motility, and invasion by *in vitro* and *in vivo* studies on gastric tumorigenesis[41].

Tumor suppressor genes can present loss of expression in GC patient samples that result in accelerated cell growth, the progression of the cell cycle, and decreased inhibition of the oncogene expression. These alterations were also studied in order to discover new diagnostic molecular markers for the early detection and progression of GC[31].

Using a gene microarray analysis, one study identified transmembrane protein with EGF like and two follistatin-like domains 2 (*tmeff2*) as a gene with significantly decreased expression in GC tissues, negatively correlated with the advanced cancer stage, large tumor size, and poor prognosis. The authors showed that the increase of *tmeff2* expression decrease cell proliferation by increasing apoptosis and by blocking the cell cycle in GC cells[42]. Moreover, modification of *tmeff2* expression in GC seems to be associated with *H. pylori* infection *via* STAT3 activation[43].

An interesting possible biomarker is gastrokine 1 (GKN1), a small protein significantly expressed in the surface lumen epithelial cell layer of gastric tissue, being involved in the maintenance of mucosal integrity and secreted into the stomach, but absent in GC[44]. It was also detected that GKN1 acts as a tumor suppressor and a modulator of apoptotic signals in GC, its lower expression might be considered an indicator of increased risk of gastric carcinogenesis[45].

Another study suggested the opportunity of detecting GC using the gene

expression profile of the blood. In this study, a four-gene panel discriminated GC with an accuracy of 95%, sensitivity of 92% and specificity of 96%. This four-gene panel for detection of GC includes two overexpressed genes: purine-rich element binding protein B (*purb*) and structural maintenance of chromosomes 1A (*smc1l1*), and two underexpressed genes: DENN/MADD domain containing 1B (*dennd1b*) and programmed cell death 4 (*pdcd4*)[46].

Next-generation deep sequencing was used to evaluate mutations of *tp53* in tumor biopsies, plasma and stomach fluids (gastric wash) obtained from GC patients. The results showed that *tp53* mutations were identified in 15/46 biopsies (32.6%), 7/46 gastric wash - (15.2%) and 6/46 plasma samples (13%). The authors suggested that gastric wash could be useful to detect DNA alterations using a comprehensive gene-panel designed for GC diagnosis[47].

METHYLATION PATTERN OF GC

In GC, epigenetic alteration by methylation occurs in specific genes involved in various processes such as cell cycle regulation (*p16nk4a, tcf4*), DNA repair (*hmlh1* and *mgmt*), cell growth/differentiation (*hoxd10, hai-2/spint2, ndrg2*), transcriptional regulation (*hltf, pax6, znf545, runx3*), cell adhesion/invasion/migration (*cdh1, cdh4, apc, flnc, lox, timp3, tsp1*), apoptosis (*bnip3, xiap, bnip3, bcl2, cacna2d3, dapk, gpx3, pcdh10, pcdh17, casp8, xaf1*), angiogenesis (*thbs-1* and *p73*), STAT pathway (*socs-1*), Ras pathway (*rassf1a, rassf2, hdab2ip, rkip*), Wnt pathway (*dkk-3, ctnnb1*), as well as in multidrug resistance genes (*mdr1, gstp1*)[48,49] and in genes associated with Epstein-Barr virus-type tumors (*pycard, bmpr1a*, and *pgr*) or *H. pylori* positive tumors (*brinp1, epha5, fli1*, and *sez6l*)[50]. The correlation of these biomarkers with tumor size, localization, differentiation, invasion, lymph node metastasis, distant metastasis, TNM stage, and prognosis is presented in Figure 2.

It was demonstrated previously that, in the case of gastric tumors, aberrant DNA methylation occurs more frequently than mutations[51], making DNA methylation a more specific assay in detection of such disease. Therefore, researchers started looking for an easier and less invasive method for the collection of cells and detection of DNA originated from gastric tumors. In serum/plasma DNA obtained from GC patient was observed a significantly higher methylation level of some biomarkers, such as *p16, cdh1, mgmt, rarb*, and *rnf180*[52].

Previously it was considered that DNA is denatured by stomach acidity[53], later on, it was demonstrated that this process is true in case of normal cells, but incorrect in case of DNA from tumor cells[45]. Collection of samples from stomach wash during endoscopy demonstrated that cancer cells from mucosal layers are easier exfoliated than normal cells into gastric juice and also that DNA isolated from such tumor cells is less degraded due to acidity[45] making it easy to be studied, offering a sensitive and quantitative method of detection.

Several genes were found to be methylated with higher frequency in gastric neoplasia versus normal condition and therefore were analyzed as possible biomarkers. Among them six methylated genes were most specific and sensitive for GC: *adam23, mint25, gdnf, prdm5, mlf1* and *rora*. The results have shown that the combination of the markers *mint25* + *adam23* + *gdnf* achieved a high sensitivity (95%) and specificity (92%). It was found that the methylation process is gene- and tumor stage-dependent during gastric carcinogenesis, some genes are highly methylated during dysplasia and early cancer phase compared with normal, but show lower methylation in advanced GC, similar with mechanism observed in ulcerative colitis-associated colon neoplasia[45].

But increased methylation process could have other causes as well, such as chronic inflammation of gastric mucosae, especially by *H. pylori* infection and aging. In order to test the effect of inflammation on methylation, the BarH-like 2 homeobox protein (*barhl2*) gene was chosen since is an *H. pylori*-independent biomarker. The *barhl2* methylation analysis of exosomal DNA (exoDNA) derived from gastric juice proven that the process is not influenced by atrophy of the gastric mucosa or *H. pylori* infection and could be used as a biomarker for detection of both early and advanced GC[54].

MIRNAS AS DIAGNOSTIC BIOMARKERS FOR GC

MiRNAs represent a class of small non-coding RNAs (19-25 nucleotides) involved by epigenetic mechanisms in many cellular processes, such as differentiation, proliferation, and apoptosis. These molecules, that seem to present specific expression

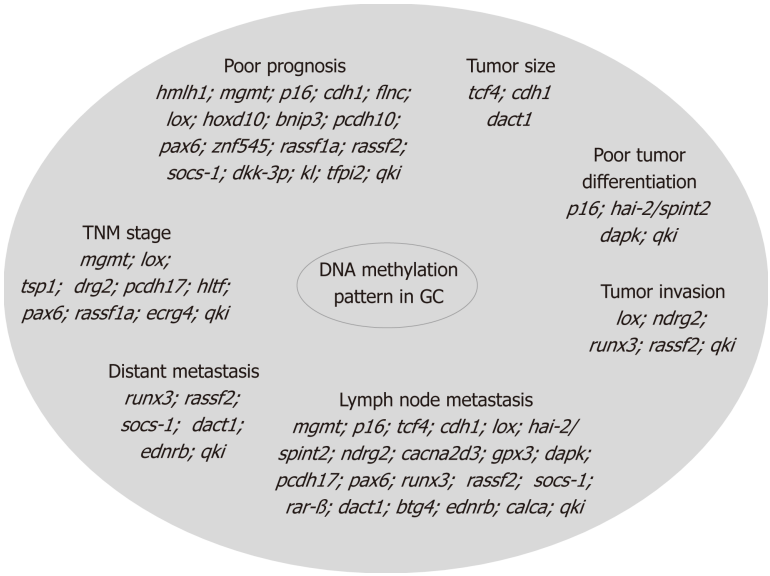

Figure 2 Methylation changes in gastric cancer. Epigenetic alteration by methylation occurs in specific genes involved in various processes such as cell cycle regulation, DNA repair, cell growth/differentiation, transcriptional regulation, cell adhesion/invasion/migration, apoptosis, angiogenesis, as well as in multidrug resistance genes, and in genes associated with Epstein-Barr virus-type tumors or *Helicobacter pylori* positive tumors. These gene alterations are correlated with tumor size, localization, differentiation, invasion, lymph node metastasis, distant metastasis, TNM stage, and prognosis. GC: Gastric cancer.

signatures in normal and tumor gastric tissue, can act as oncogenes and/or tumor suppressors depending on the role of the target mRNA/gene[55].

More studies suggested that miRNAs could be considered important potential biomarkers for gastric pathology as they are frequently found to be deregulated in gastric tissue in *H. pylori* infection, chronic gastritis, preneoplastic conditions such as atrophic gastritis and intestinal metaplasia, and also in early dysplasia and invasive cancer. Moreover, modifications of miRNA blood levels were also identified in GC patients supporting the development of new diagnostic and prognostic methods based on miRNA expression analysis[56].

A promising result was obtained by a study in which miRNA-21 levels, in serum and peripheral blood mononuclear cells, were found to be increased in GC patients with a positive prediction rate around 90%, while those of CA199 and CEA were around 50%. Moreover, circulating miR-21 levels can discriminate between stage I and stage IV of GC[57].

miR-376c was found to be up-regulated in tissue, plasma, and urine of GC patients, even from the early stage of the tumor. The increased expression of miR-376c was associated with the proliferation, migration and anchorage-independent growth of cancer cells, having as a direct target *arid4a* gene which is considerably down-regulated in tumor tissue[58].

Increased pre-operative circulating miR-196a and miR-196b levels were identified in GC patients compared to healthy controls, the expression level of these miRNAs being reduced after the surgical resection of the gastric tumor. Interestingly, higher circulating miR-196a/b levels were correlated with the metastatic potential of the tumor, advanced stages, and poorer survival. Moreover, the results of this study suggested that circulating miR-196a, miR-196b, and combined miR-196a and miR-196b can distinguish between GC patients and healthy controls with higher sensitivity and specificity compared to the CEA or CA19-9[59]. Another recent study analyzed circulating miRNA levels in GC patients and identified a four-miRNA panel (miR-501-3p, miR-143-3p, miR-451a, miR-146a) as possible noninvasive biomarkers for prediction and prognosis of lymph node metastasis (LNM). In addition, LNM patients with decreased levels of miR-451a and miR-146a presented worse overall survival[60]. A five-miRNA panel (miR-16, miR-25, miR-92a, miR-451, and miR-486-5p) was found to be differentially expressed in plasma of gastric non-cardia adenocarcinoma patients compared to healthy controls. This panel seems to be able to discriminate between early-stage of gastric non-cardia adenocarcinoma patients and cancer-free subjects[61]. Other panels containing up-regulated miRNAs (miR-200a-3p, miR-296-5p, miR-132-3p, miR-485-3p, and miR-22-5p)[62] and (miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, and miR296-5p)[63] were identified in serum of the GC patients compared to healthy controls. Based on the evidence that exosomes secreted by cancer

and normal cells can be released into the circulatory system, a recent study identified overexpression of circulating exosomal miR-19b and miR-106a in GC patients compared to healthy controls. These increased levels were correlated with lymphatic metastasis and advanced stages of GC[64].

miR-146, miR-375, and Let-7 were found to be downregulated while miR-19 and miR-21 presented an increased expression in plasma of the GC patients with *H. pylori* infection. The study also identified overexpression of the genes involved in IRAK4 signaling and a decreased expression of *pten* gene in the GC patients with *H. pylori* infection compared to the control group, suggesting the potential of these molecules as biomarkers for early diagnosis of GC[65]. There are also other molecular potential biomarkers for screening GC identified in gastric juice: miR-421, miR-21, miR-106a and miR-129[66].

Table 1 summarized several miRNAs presenting modified circulating expression in GC patients compared to healthy controls.

Even if these results need to be validated by independent groups or cohorts in prospective studies, circulating miRNAs could be considered a class of novel, non-invasive diagnostic biomarkers with sufficient diagnostic accuracy in detecting the early-stage GC.

LNCRNAS IN GC

LncRNAs are transcripts longer than 200 nucleotides with no or limited protein-coding potential. lncRNAs are implicated in the regulation of several biological processes like transcription and translation, cellular differentiation, gene expression, cell cycle, *etc*[71]. They are characterized by high stability while circulating in body fluids and their level in tumor tissue correlates with plasma levels. As such, lncRNAs can be used to distinguish tumor patients at early stages from healthy people, as well as to predict the prognostic, metastasis risks and recurrence after surgery[72,73].

In 2013, Cao *et al*[74] investigated the lncRNA expression in GC and detected 88 differentially expressed lncRNAs, 71 upregulated and 17 downregulated. Zhou *et al*[75] hypothesized that GC-related lncRNAs might be released into the circulation during tumor initiation and could be utilized to detect and monitor GC.

Highly upregulated in liver cancer (HULC) is a lncRNA implicated in the growth and tumorigenesis of human GC. *In vitro* overexpression of HULC in gastric cell lines stimulates proliferation and invasion, inhibits cell apoptosis and can induce autophagy patterns, while its silencing reverses the EMT phenotype[76]. Evaluated in plasma, HULC level is higher in preoperative patients compared with healthy control subjects[77].

Another candidate as a possible biomarker for early detection and prognosis prediction of GC is lncRNA PVT1 since the levels of PVT1 in gastric juice from gastric patients were significantly higher than those from normal subjects[78].

Zhou *et al*[75] proposed H19 (imprinted maternally expressed transcript) as a potential biomarker for diagnosis of GC, especially for early tumor screening. It stimulates cell proliferation and inhibits apoptosis[79]. H19 plasma level is significantly higher in GC patients compared with normal controls[75,80-82] and allows the discrimination of early stage GC[75]. On the other side, H19 plasma levels were significantly lower in postoperative samples than in preoperative ones[75,80]. Also, patients with smaller tumor sizes (< 5 cm) exhibit higher H19 level in their circulation compared with those with larger tumors (≥ 5 cm)[81].

Another abnormally expressed lncRNA in GC is long intergenic non-protein-coding RNA 152 (LINC00152), its plasma level being significantly elevated in GC patients compared with healthy controls[83,84] and presenting higher levels in postoperative plasma samples compared with preoperative ones[83]. This lncRNA allows differentiating GC patients from ones with benign gastric diseases and can be also detected in gastric juice[84]. Another lncRNA that can be detected in gastric juice is AA174084 characterized by higher levels in GC patients compared with healthy or other non-GC subjects. Its plasma level drops markedly in GC patients on day 15 post-surgery and is associated with invasion and lymphatic metastasis[85].

Hox transcript antisense intergenic RNA (HOTAIR) has been suggested to be implicated in GC tumorigenesis and progression[86]. It promotes cell proliferation and inhibits apoptosis[79]. HOTAIR plasma level is significantly higher in GC patients compared with healthy controls. Moreover, increased HOTAIR expression was associated with advanced tumor stages, higher grades, and metastasis[86]. Other up-regulated lncRNAs are human urothelial carcinoma associated 1 (UCA1), which is implicated in GC carcinogenesis and presents higher levels in GC patients[87], and ABHD11-AS, whose levels in gastric juice is significantly higher in GC patients, being

Table 1 Dysregulated circulating microRNAs reported in gastric cancer patients

MicroRNA	Biological function	Type of biomarker	Origin of specimen	Sensitivity/specificity	Study
miR-21	Upregulated; discriminates between stage I and stage IV of GC	Diagnostic, prognostic	Serum, PBMC	88.4%/79.6% (serum) 81.3%/73.4% (PBMC)	Wu *et al*[57]
miR-196a/b	Upregulated; correlated with metastatic potential of the tumor, advanced stages, and poorer survival	Diagnostic, prognostic	Plasma	69.5%/97.6%	Tsai *et al*[59]
miR-200c	Upregulated; predictor of progression and survival.	Diagnostic, prognostic	Blood	65.4%/100%	Valladares-ayerbes *et al*[67]
miR-940	Downregulated	Diagnostic	Plasma	81.25 %/98.57 %	Liu *et al*[68]
miR-551b-5p	Upregulated	Diagnostic	Serum	77.5%/80.0%	Jiang *et al*[69]
miR-19b, miR-106a	Upregulated; correlated with lymphatic metastasis and advanced stages	Diagnostic, prognostic	Circulating exosomes	95%/90%	Wang *et al*[64]
miR-501-3p, miR-143-3p, miR-451a, miR-146a	Differentially expressed; prediction and prognosis of lymph node metastasis	Prognostic	Serum	87.78%/63.33%	Jiang *et al*[60]
miR-16, miR-25, miR-92a, miR-451, miR-486-5p	Differentially expressed; discriminate between early-stage of GC and cancer-free subjects	Diagnostic	Plasma	72.9%/89.2%	Zhu *et al*[61]
miR-200a-3p, miR-296-5p, miR-132-3p, miR-485-3p, miR-22-5p	Upregulated	Diagnostic	Serum	N/S	Wang *et al*[62]
miR10b-5p, miR132-3p, miR185-5p, miR195-5p, miR-20a3p, miR296-5p	Upregulated	Diagnostic	Serum	N/S	Huang *et al*[63]
miR-17-5p, miR-21, miR-106a, miR-106b, let-7a	Differentially expressed	Diagnostic	Plasma	85.5%/80.0%	Tsujiura *et al*[70]
miR-146, miR-375, let-7, miR-19, miR-21	Differentially expressed in GC patients with *H. Pylori* infection	Diagnostic	Plasma	N/S	Ranjbar *et al*[65]

miR-: MicroRNA; GC: Gastric cancer; PBMC: Peripheral blood mononuclear cell; *H. pylori*: *Helicobacter pylori*; N/S: Not specified.

also associated with clinicopathological factors[88].

Yang *et al*[89] investigated the diagnostic value of gastric cancer associated transcript 2 (GACAT2) in GC. In the evaluated cohort, the plasma GACAT2 levels in GC patients were significantly higher compared with healthy individuals, as well as in the preoperative group compared with the postoperative one. In addition, the individual relative changes of GACAT2 expression following surgery were significantly associated with lymphatic metastasis, distal metastasis, and perineural invasion.

Also, some lncRNAs panels were investigated for GC diagnosis. Zhang *et al*[90] identified a panel of five novel plasma lncRNAs (TINCR, CCAT2, AOC4P, BANCR, and LINC00857) using genome-wide lncRNA screening analysis which could distinguish GC patients from healthy controls and can help monitor tumor dynamics, tumor, depth of invasion, lymphatic metastasis and more advanced tumor stages. Also, Dong *et al*[91] identified a three-lncRNA signature, CUDR, LSINCT-5, and PTENP1, that allows distinguishing healthy controls from early GC patients.

However, to introduce lncRNAs as plasma biomarkers, further studies and improvements of extraction, quantification, probe enrichment, and evaluation methods should be performed.

CIRCRNAS, A NEW CLASS OF GC BIOMARKERS

CircRNAs are a new class of non-coding RNAs that form a closed loop, without 5′ and

3′ ends[92]. CircRNAs were first identified in RNA viruses, but later with the progress of new molecular techniques like high-throughput RNA sequencing and microarray analysis, circRNAs were found in all eukaryotic organisms as stable and conserved sequences that control gene expression through interactions with miRNAs[93]. New emerging data have confirmed that circRNAs are involved in the occurrence of many diseases, and also are strongly associated with tumor growth and metastasis[94]. These findings underline the potential of circRNAs to act as novel biomarkers and therapeutic targets for various human tumors.

Several recent studies have analyzed the aberrant expression of circRNA in GC compared with adjacent normal tissue and presented various lists with upregulated and downregulated circRNA[95-98] (Table 2). The study performed by Huang *et al*[96] identified circRNA0026 (hsa_circ_0000026) as having significantly downregulated expression 2.8fold change in GC. Sui *et al*[95] found six differentially expressed circRNA in GC tissue and managed to validate through qRT-PCR three of them (hsa_circRNA_400071, hsa_circRNA_000543, and hsa_circRNA_001959) as having a consistent expression with the differentially expressed gene. Through analysis of circRNA and mRNA differential expression profiles in GC tissues, the authors managed to identify the target mRNA and their respective genes for selected circRNAs, like *cd44, cxxc5, myh9, malat1* and other genes with important implications in GC tumorigenesis and development.

One important finding related to circRNA in cancer was that they are not easily degraded by RNase and thus, are stably expressed in human cells, in plasma or in gastric juice[104,109]. These findings opened the way for plasma circRNA profiling studies, aiming to identify specific diagnostic and prognostic circRNA for GC patients. Li *et al*[108] performed circRNA microarray for three GC samples and plasma, to assess the differences of circRNA expression profiles. They found that 3 and 14 circRNA were upregulated and downregulated respectively, both in patients' plasma and tumor tissue. Further, they analyzed through RT-droplet digital PCR (RT-ddPCR) the circRNA levels in plasma for 121 GC patients. Two circRNA: hsa_circ_0001017 (30.85-folds change) and hsa_circ_0061276 (121.54-folds change) were selected for their non-invasion diagnostic values. Results showed that patients with low level of hsa_circ_0001017 or hsa_circ_0061276 in plasma had shorter overall survival than those with high levels. Moreover, patients whose plasma levels of the two circRNA recovered to normal after the operation had longer disease-free survival.

Due to their documented correlation between tissue and plasma level, stability and presence as cell-free RNA in plasma, circRNA may be valuable blood-based biomarkers for GC screening, diagnosis, and prognosis.

CIRCULATING TUMOR CELLS

GC diagnosis relies mostly on invasive procedures, which are rather expensive and may have sometimes serious adverse events[110]. In spite of being documented 150 years ago[111], only last years proved that analyzing circulating tumor cells (CTCs) in liquid biopsies, a blood-based diagnostic approach, as a substitute for tissue biopsies have emerged as real-time cancer development monitoring tool and management strategy[112].

CTCs are a very rare and heterogeneous population of cells circulating in peripheral blood, originating from either primary or metastatic tumors that express the antigenic or genetic characteristics of the specific tumor type[113]. CTCs were first described as expressing epithelial cell markers EpCAM, cytokeratin 8, 18, and 19 (CK8, CK18, CK19), and are CD45 negative[114]. Recently, EMT with potential overexpression of mesenchymal markers and decreased expression of epithelial cell markers or mesenchymal-epithelial transition (MET) that present mesenchymal and epithelial markers, were shown to characterize subpopulations of these cells[115,116]. Mesenchymal phenotypes have larger plasticity thus facilitating migration, invasion, and drug resistance[117]. Several studies revealed the presence of CTCs in circulating tumor microemboli (CTM), indicating poor prognosis and influencing disease progression[118].

This high heterogeneity of CTCs prompted researchers to develop different methodologies to enrich, isolate and/or enumerate them based on specific phenotypic or molecular characteristics. Basically, there are two general types of methods used in CTCs enrichment/isolation: biological and physical methods. Their combination is more likely to improve the efficiency of CTC detection. CellSearch™ platform (Veridex LLC, Huntingdon Valley, PA, United States) the only procedure approved for the enumeration and isolation of CTCs by the Food and Drug Administration (FDA) for clinical use, detect the adhesion molecule EpCAM, CK8, CK18 and CK19

Table 2 Various aberrantly expressed circular RNAs with the potential to serve as diagnostic and prognostic biomarkers for gastric cancer

circRNA name	Biological function	Type of biomarker	Origin of specimen	Sensitivity/specificity	Study
hsa_circ_002059	Downregulated in GC; correlated with TNM stage and metastasis	Diagnostic	Tissues; plasma	81%/62%	Li *et al*[94]
hsa_circ_0000096	Downregulated in GC; affects GC cell growth and migration	Diagnostic	Tissues	N/S	Li *et al*[99]
hsa_circ_0058246	Upregulated in GC; associated with poor clinical outcomes	Prognostic	Tissues	N/S	Fang *et al*[100]
hsa_circ_0000745	Downregulated in GC; correlated with tumor differentiation and tumor nodal metastasis	Prognostic	Tissues	85.5%/45%	Huang *et al*[101]
hsa_circ_00000181	Downregulated in GC; associated with TNM stage and metastasis	Prognostic	Plasma	99%/85.2%	Zhao *et al*[102]
hsa_circ_0047905, has-circRNA7690-15, hsa_circ_0138960	Substantially upregulated in GC; act as tumor promoters in the pathogenesis of GC	Diagnostic	Tissues	N/S	Lai *et al*[103]
hsa_circ_0014717	Downregulated in GC; stably expressed in gastric juice; associated with TNM stage and metastasis	Prognostic	Tissues	59.38%/81.25%	Shao *et al*[104]
hsa_circ_0001895	Downregulated in both GC tissue and gastric precancerous lesions	Diagnostic	Tissues	67.8%/85.7%	Shao *et al*[105]
has_circ_0000520	Downregulated in GC; associated with TNM stage and in GC plasma linked with CEA expression	Diagnostic	Tissues; plasma	53.57%/85.71% (tissue) 82.35%/84.44% (plasma)	Sun *et al*[106]
hsa_circ_0000190	Downregulated in GC; associated with TNM stage and metastasis	Diagnostic	Tissues; plasma	71.2%/75%	Chen *et al*[107]
hsa_circ_0001017 hsa_circ_0061276	Downregulated in GC; associated with shorter overall survival	Prognostic	Tissues; plasma	95.5%/95.7%	Li *et al*[108]

GC: Gastric cancer; CEA: Carcinoembryonic antigen; circRNA: Circular RNA; N/S: Not specified.

and exclude CD45 cells but may overlook CTCs with predominantly mesenchymal phenotype. Using cell size - and phenotype-based systems, as centrifugal microfluidic system based on fluid-assisted separation technique (FAST), or Cascaded Inertial Focusing Microfluidic device, coupled with detection of an extended panel of markers might identify a different subpopulation of CTCs with higher efficiency[119,120].

Exploiting a frequent genetic abnormality reported in GC tumors, the aneuploidy of chromosome 8, Li *et al*[121] created an integrated subtraction enrichment (SET) and immunostaining-fluorescence in situ hybridization (iFISH) platform claimed to be more sensitive than the CellSearch™ to detect and characterize CTCs in advanced GC patients. Multiple studies showed that SET-iFISH method to enumerate CTCs with chromosome 8 aneuploidy is efficient in monitoring GC patient treatment response[113]. Expression of different other markers as vimentin, twist, MUC1, HER2, *etc*. proved to be very useful to evaluate therapeutic response and prognosis in patients with GC. However, irrespective of the detection method employed, there is weak evidence that detection of CTCs has the potential for early biomarker detection in GC but all data are consistent in supporting its utility in assessing the tumor heterogeneity, monitoring treatment responses and real-time cancer management[113].

CIRCULATING TUMOR DNA

Circulating tumor DNA (ctDNA) analysis refined the liquid biopsy to the level of identification of tumor molecular traces circulating in the body fluids and may give deeper insight on the cancer heterogeneity, early biomarker detection, therapeutic target detection, real-time evaluation of treatment response and possible resistance and prognosis. Originating from primary tumor cells, CTCs and/or distant metastasis, ctDNA give a broad cross-section of the disease offering information on methylation status, genetic alterations as mutations, amplifications, rearrangements, copy number variation (CNV), the latter being more difficult to analyze due to the short length and possibly unequal distribution of the ctDNA fragments[111].

Generally, ctDNA represents only a fraction of the cell-free circulating DNA (cfDNA), which is increased considerably in late-stage disease[122]. However, there is evidence that ctDNA can be detected in the plasma of cancer patients even in the early stages of their disease[123,124]. In GC, Fang *et al*[125] found that ctDNA levels were correlated with vascular invasion and the highest ctDNA detectable levels were associated with peritoneal recurrence and a poor prognosis. Balgkouranidou *et al*[126] showed that *rassf1a* and *apc* promoter hypermethylation in cfDNA represents a frequent epigenetic event in patients with early operable GC demonstrating a prognostic capacity for these patients. Another study suggested that cfDNA can identify EBV-associated gastric carcinoma (EBVaGC) subtype and monitor tumor progression as well as treatment response in patients with EBVaGC[127].

Being a rare event, ctDNA requires highly sensitive and reproducible analytical methods for proper investigation. Multiplex mass spectrometric SNP genotyping technology, real-time quantitative PCR (qRT-PCR), digital droplet PCR (ddPCR) with improved nucleic acid quantification, next-generation sequencing (NGS) were already employed for ctDNA analysis in GC patients[125,128-130] proving the usefulness in personalized treatment decisions. A panel of more than 70 genes and genomic biomarkers for MSI and blood tumor mutational burden (bTMB) by Foundation Medicine, the FoundationACT® assay, was granted breakthrough device designation by the FDA[131] and might become the first FDA-approved liquid biopsy assay to incorporate multiple companion diagnostics (CDx) and multiple biomarkers.

CONCLUSION

GC remains an important cause of cancer death worldwide with a high mortality rate due to the fact that the majority of GC cases are diagnosed at an advanced stage when the prognosis is poor and the treatment options are limited. Unfortunately, the existing circulating biomarkers for GC diagnosis and prognosis display low sensitivity and specificity and the GC diagnosis is based only on the invasive procedures such as upper digestive endoscopy. Therefore, most current GC studies are focused on the identification and validation of non-invasive cancer biomarkers released from the tumor tissues into the body fluids, such as blood and stomach juice. Many of these biomarkers are not specific for the early stages, being detected in advanced stages of GC, and cannot be used for early GC detection. However, some of recently discovered circulating molecules (miRNAs, lncRNAs, circRNA) hold the promise for developing new strategies for early diagnosis of GC, being able to discriminate between the early stage of GC and healthy subjects, with a sensitivity more than 77.5%. In order to improve the sensitivity and enlarge the early stage biomarkers list, further studies should be performed to optimize laboratory techniques such as extraction, quantification, probe enrichment, and evaluation methods. Moreover, these results need to be validated by independent groups or cohorts in prospective studies.

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World Journal of Gastroenterology

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World J Gastroenterol 2019 May 7; 25(17): 2045-2057

DOI: 10.3748/wjg.v25.i17.2045

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Evolving screening and surveillance techniques for Barrett's esophagus

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Author contributions: Steele D was responsible for the literature review, analysis, interpretation and drafting of the article; Kyanam Kabir Baig K was responsible for additional design, critical revision of the article for important intellectual content; Peter S was responsible for conception and design, critical revision and final approval of article.

Conflict-of-interest statement: No potential conflicts of interest. No financial support.

Manuscript source: Invited manuscript

Received: January 21, 2019
Peer-review started: January 21, 2019
First decision: February 21, 2019
Revised: March 26, 2019

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Abstract

Barrett's esophagus (BE) is a change in the esophageal lining and is known to be the major precursor lesion for most cases of esophageal adenocarcinoma (EAC). Despite an understanding of its association with BE for many years and the falling incidence rates of squamous cell carcinoma of the esophagus, the incidence for EAC continues to rise exponentially. In association with this rising incidence, if the delay in diagnosis of EAC occurs after the onset of symptoms, then the mortality at 5 years is greater than 80%. Appropriate diagnosis and surveillance strategies are therefore vital for BE. Multiple novel optical technologies and other advanced approaches are being utilized to assist in making screening and surveillance more cost effective. We review the current guidelines and evolving techniques that are currently being evaluated.

Key words: Barrett's esophagus; Screening; Surveillance; New techniques; Endoscopy; Imaging; Radiofrequency ablation; Narrow band imaging

Core tip: Appropriate screening and diagnosis of Barrett's esophagus and dysplasia and thereby cancer prevention is challenging. Newer imaging modalities aid and complement the role of traditional endoscopy with biopsy. Research in this area is promising and primarily focused on improved optical technology and advanced sampling techniques.

Citation: Steele D, Baig KKK, Peter S. Evolving screening and surveillance techniques for Barrett's esophagus. *World J Gastroenterol* 2019; 25(17): 2045-2057
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2045.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2045

Accepted: April 10, 2019
Article in press: April 11, 2019
Published online: May 7, 2019

P-Reviewer: Espinel J, Herbella F, Kariv R, Lei YC, Mastracci L, Savarino V, Shiryajev YN
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

RATIONALE FOR SCREENING

Barrett's esophagus (BE) is characterized by the replacement of squamous epithelium normally found in the esophagus with metaplastic columnar epithelium. As a result, the proximal level of the squamocolumnar junction, also known as the z-line, no longer corresponds with the gastro-esophageal junction (GEJ). This change is a result of chronic exposure of the normal squamous epithelium to refluxed gastric material and is believed to increase the risk of evolution to neoplasia[1].

The index examination is essential for identifying and diagnosing BE as this ultimately determines follow up intervals moving forward. Using white light endoscopy (WLE), BE can be accurately visualized and then divided into short segment (shorter than 3 cm) and long segment (longer than 3 cm). Endoscopically, BE is then further graded using Prague C and M criteria. The Prague C and M criteria is a validated grading system that assesses the presence and extent of BE by measuring the circumferential (C) length and maximum (M) length of BE visualized above the GEJ[1]. Once BE has been recognized and graded, biopsies are then obtained using the Seattle protocol. The Seattle protocol is a technique that aims to identify BE with or without dysplasia and neoplasia by obtaining 4-quadrant biopsies every 1-2 centimeters within this area of identified BE. In addition to Seattle protocol, biopsies are obtained of any areas of mucosal irregularity. These biopsies are then sent for pathology where the diagnosis of BE or is confirmed by the identification of intestinal metaplasia on biopsy[1,2]. Since dysplastic and neoplastic lesions can be dispersed throughout a segment of BE, sampling error decreases the sensitivity of random biopsies using the Seattle protocol, especially for segments longer than 3 cm[3].

While this change in the esophageal lining is largely asymptomatic, BE is known to be the major precursor lesion for most cases of esophageal adenocarcinoma (EAC). Esophageal cancer is the eighth most common cancer in the world with approximately 10000 new cases of EAC diagnosed every year. Despite an understanding of its association with BE for many years and the falling incidence rates of squamous cell carcinoma of the esophagus, the incidence for EAC continues to rise exponentially[4]. In association with this rising incidence, if the delay in diagnosis of EAC occurs after the onset of symptoms, then the mortality at 5 years is greater than 80%. Alternatively, if EAC is diagnosed at an early stage, T1a, then the 5-year mortality is significantly better at greater than 80%[5]. Given this rising incidence and poor prognosis from EAC which has a known precursor lesion in BE that can be endoscopically monitored, significant interest has been placed in finding an effective way to accurately screen for BE.

CURRENT RECOMMENDATIONS

Whereas there is significant concern for the rising incidence in EAC, screening is limited to a very specific patient population. Some of the limitations to screening the general population include the lack of an accurate, widely applicable risk assessment tool, lack of a cost-effect screening method and the absence of a beneficial effect on mortality. Additionally, the incidence of EAC is rising, the absolute risk of developing EAC in the setting of having BE remains low. The most recent data has shown the prevalence of BE in the general population to be around 1%-2% and the annual risk of BE converting to EAC between 0.12%-0.5%[4]. For these reasons, screening the general population for BE by endoscopic or non-endoscopic methods is not advocated. Although screening the general population may not be recommended at this time, screening targeted populations is encouraged.

BEST PRACTICES

While recommendations amongst the major gastroenterological societies differ, as outlined in Table 1, the overall consensus is to screen individuals with multiple risk factors for BE/EAC, and in the case of the American College of Gastroenterology and the American Society for Gastrointestinal Endoscopy to screen patients who have been experiencing symptoms for a prolonged period of time[5]. An international consensus statement (BOB CAT) recommended endoscopic screening for men older than 60 years of age who have experienced chronic gastroesophageal reflux disease (GERD) symptoms for 10 years or longer[4].

Furthermore, attempts have been made to create a validated model to determine risk of progression of BE to neoplasia. One model has been the creation of the 'Progression in BE score or PIB score' which further outlined in Tables 2 and 3. This

Table 1 Current guidelines for screening Barrett's esophagus from major gastroenterology societies[5]

Society (year published)	Target populations
American College of Gastroenterology (2016)	Primary: Male patients with either > 5 years of GERD or with more than weekly GERD symptoms and at least two other risk factors including: (1) Age > 50; (2) central obesity; (3) smoking history; (4) Caucasian; (5) first degree relative with BE or EAC
American Society for Gastrointestinal Endoscopy (2012)	Patients with multiple risk factors including male sex, older than 50, Caucasian, family history of BE, increased duration of reflux symptoms, smoking and obesity
American Gastroenterological Association (2011)	Patients with multiple risk factors including male sex, older than 50, Caucasian, chronic GERD, hiatal hernia and obesity
British Society of Gastroenterology (2014)	Primary: Patients with GERD and at least three risk factors including male, older than 50, Caucasian, and obesity unless there is a family history of BE or EAC which would lower threshold

GERD: Gastroesophageal reflux disease; BE: Barrett's esophagus; EAC: Esophageal adenocarcinoma.

scoring system uses the risk factors identified as significant in causing the highest risk of developing high-grade dysplasia (HGD)/EAC [male sex, smoking, length of BE and baseline low-grade dysplasia (LGD)] to determine patients with BE who are at low, intermediate and high risk for HGD or EAC. Using the point system in Table 3, patients with 0-10 points were considered low risk for progression, patients with 11-20 were considered intermediate risk and patients with 21-30 were considered high risk[6]. The annual risk of progression was 0.13% for low, 0.73% for intermediate and 2.1% for high risk groups respectively[6]. Of note, this score is useful in patients who have been diagnosed and have established BE.

This targeted screening approach may assist in the diagnosis of BE/EAC in some patients, however, approximately one half of patients with EAC report never having symptoms of heartburn prior to their diagnosis. In addition, the gold standard for diagnosis, upper gastrointestinal endoscopy is invasive and expensive[4]. While BE is a known precursor lesion to EAC, studies have shown that more than 90% of EAC is diagnosed in the absence of a prior diagnosis of BE. While not entirely understood, a possible explanation for this occurrence is likely related to the disease being diagnosed at a later stage after the progression of BE to dysplasia and eventually EAC has already occurred. Importantly, this shows that we are missing a significant number of at risk patients with the current screening guidelines[7].

Given all of these considerations, finding a universally accepted screening modality program for EAC remains a challenge, however, identifying the key components will increase the likelihood for success. The primary element to developing a successful screening program is finding a screening tool that is "minimally or noninvasive, cost-effective, widely applicable, safe and accurate in the diagnosis of BE"[5]. Once this has been identified, it will also be important to recognize a validated risk assessment tool to target those at risk for developing BE/EAC as well as a tool to predict those at highest risk for progression of disease. The final phase will include finding a cost-effective tool to treat dysplasia or early EAC once diagnosed by screening or surveillance[5]. Ongoing research is being performed to address these issues to allow for widespread screening and subsequently the surveillance and treatment of BE and EAC.

EMERGING TECHNOLOGIES TO ENHANCE SCREENING OF BE

In this section, we will review the current and emerging techniques being used for the screening of BE.

Optical technologies

Conventional white light endoscopy: High-definition (HD) upper gastrointestinal endoscopy is currently used as the gold standard in screening targeted populations. HD has replaced standard definition (SD) endoscopy over the last several years due to the limited sensitivity and specificity of SD[8]. The image resolution of HD utilizes more than 1 million pixels compared to just 100000-400000 with SD. This enhances the ability to visualize subtle mucosal changes to allow for more accurate biopsies of areas concerning for BE or endoscopically suspected esophageal metaplasia[9].

Table 2 Univariate analyses for each risk factors progression to high grade dysplasia or esophageal adenocarcinoma[4]

Variable	Adjusted *P* value and hazard ratios (95%CI)
Males	*P* = 0.0023, HR = 3.01 (1.48-6.11)
Smoking	*P* = 0.0029, HR = 1.83 (1.23-2.71)
Age + 10 yr	*P* = 0.3055, HR = 0.96 (0.89-1.04)
Caucasian	*P* = 0.8429, HR = 1.06 (0.61-1.82)
Hiatal hernia present	*P* = 0.5928, HR = 1.12 (0.73-1.72)
Visible lesion at baseline	*P* = 0.9254, HR = 1.04 (0.49-2.2)
Aspirin use	*P* = 0.2807, HR = 0.81 (0.56-1.18)
Non-steroidal anti-inflammatory drug	*P* = 0.5602, HR = 0.9 (0.64-1.28)
Proton pump inhibitor	*P* = 0.8197, HR = 0.9 (0.37-2.21)
Low grade dysplasia	*P* ≤ 0.0001, HR = 3.68 (2.56-5.31)
BE length + 1 cm increase in length	*P* ≤ 0.0001, HR = 1.12 (1.08-1.18)

BE: Barrett's esophagus; HGD: High grade dysplasia; EAC: Esophageal adenocarcinoma; HR: Hazard ratio; CI: Confidence interval.

While HD WLE is the gold standard in screening and surveillance, as discussed before, cost remains the primary limiting factor for its use as a screening tool for the general population. The cost is multifactorial and includes the cost of the procedure, sedation, and the cost of upgrading entire endoscopy systems from SD endoscopy[8]. In addition to cost, concerns also exist regarding the missed rates of dysplastic or neoplastic lesions. In a study to evaluate the efficiency of biopsies using standard protocol, the missed rates were as high as 57%[8]. As such, advanced imaging technologies have started to emerge to enhance the screening, surveillance, and treatment of patients with BE[8].

Chromoendoscopy: Chromoendoscopy is a technique where stains are applied topically to enhance mucosal visualization during upper endoscopy. The goal of this approach is to improve visualization of the mucosa and the vascular pattern of absorption to improve detection of abnormalities and target biopsies[9]. The most commonly used stains include indigo carmine, methylene blue, and acetic acid[1,4].

Indigo carmine is a non-absorptive contrast dye frequently used in conjunction with magnification endoscopy to identify irregular mucosa and pit patterns seen within segments of BE[1,9]. These mucosal findings have been shown to correlate with presence of intestinal metaplasia and dysplasia. Methylene blue is a chemical that can be absorbed by intestinal epithelium without being absorbed by squamous or gastric epithelium. When compared to traditional surveillance techniques, several studies have shown methylene blue could discern areas of intestinal metaplasia and dysplasia with high accuracy and fewer biopsies[9]. Despite these studies, overall, traditional techniques have been shown to be non-inferior which in conjunction with a potential risk for carcinogenesis from methylene blue, the widespread use of chromoendoscopy using methylene blue has been limited[9].

Acetic acid in combination with either HD or magnification endoscopy works to provide contrast enhancement of the mucosa. Initial application of acetic acid turns all of the esophageal and gastric mucosa white. After several minutes, however, while the normal mucosa remains white, gastric columnar mucosa and areas of BE will turn red[1]. Multiple studies looking at patients undergoing surveillance for BE have shown that targeted biopsies with acetic acid chromoendoscopy yield higher rates of detection of dysplasia and neoplasia while fewer biopsies are required[1].

Lugol's solution or more commonly known as Lugol's Iodine (LI) is a compound stain that contains iodine and potassium iodide that when absorbed by the squamous mucosa of the esophagus, stains it brown. By staining the squamous epithelium brown, LI highlights any metaplastic columnar epithelium within the esophagus[10,11]. One small study evaluated the accuracy of diagnosing BE using LI in 11 subjects with known columnar epithelium in the esophagus and compared them with 12 control subjects. The sensitivity and specificity of of diagnosing BE using LI was 89% and 93% respectively[12].

Overall, the advantage of chromoendoscopy is that it is relatively inexpensive and the chemical solutions are readily available for use. There are however several disadvantages to using chromoendoscopy. The biggest disadvantage is high inter-observer variability in ability to identify abnormal mucosa. Additionally,

Table 3 Progression in Barrett's esophagus point system based on risk variables[4]

Variable	Points
BE length in centimeters	
< 1	0
1 to < 2	1
2 to < 3	2
3 to < 4	3
4 to < 5	4
5 to < 6	5
6 to < 7	6
7 to < 8	7
8 to < 9	8
9 to < 10	9
10 +	10
Males	9
Smokers	5
Baseline confirmed LGD	11

BE: Barrett's esophagus; LGD: Low grade dysplasia.

chromoendoscopy can be labor intensive requiring a separate catheter and often multiple sprays in order to adequately visualize the mucosa[1,4,9].

Electronic chromoendoscopy: Electronic chromoendoscopy generally refers to endoscopic imaging technologies that enhance the mucosal surface and blood vessels through contrast enhancement[1]. This section will review four processor enhanced electronic systems: Narrow band imaging (NBI), Fuji Intelligent Chromoendoscopy (FICE), i-SCAN, and blue light imaging (BLI).

NBI (Olympus Evis Exera System®) differs from chromoendoscopy in that no stains are used. Instead, NBI works by enhancing the resolution of the mucosal surface by restricting the range of wavelengths of light. Several meta-analysis studies have shown NBI to do well in detecting HGD with high sensitivity (96%) and specificity (94%) in one study while using fewer biopsies when compared to WLE. At the same time, several studies have been unable to show a difference in detecting neoplasia when compared to WLE[4,9] (Figures 1-3). The advantages to NBI are that it is relatively cheap; widely available given it is integrated in most standard equipment and its ease of use[9]. NBI also has the advantage over dye-based chromoendoscopy because there is no risk for potential toxicity[4]. Previously, a disadvantage of NBI has been the lack of a universal classification system; however, in 2016, the Barrett's International NBI Group aimed to develop and validate a classification system to identify dysplasia and EAC in patients with BE using NBI. The BING criteria were created by a group of experts in NBI who reviewed images of non-dysplastic BE, dysplastic BE, and EAC to characterize the different mucosal and vascular patterns using NBI (Table 4). Using these criteria, patients undergoing surveillance/treatment of BE were then recruited to obtain high resolution NBI images and biopsies for histologic review. Using the newly formed BING criteria, the NBI images from these patients were reviewed by experts to determine the validity of the BING criteria for accuracy. The results from this study found that the BING criteria identified patients with dysplasia with an overall accuracy of 85% and when dysplasia identified with a high degree of confidence, the overall accuracy was 92% with a high level of inter-observer agreement[13].

FICE (FUJIFILM Endoscopy System®) manipulates certain ranges of wavelengths (red, green and blue) to yield a color-enhanced image of the superficial mucosa and vascular structures in real time. A study comparing FICE to acetic acid chromoendoscopy found FICE to have comparable sensitivity in detecting neoplasia in BE[9]. At this time, however, more data is needed to assess the right setting for tissue diagnosis in FICE.

Similar to FICE, i-SCAN (PENTAX Endoscopy System®) is software driven electronic chromoendoscopy technique that manipulates wavelengths to produce an enhanced image. Limited data also exists for i-SCAN. In a randomized trial comparing standard protocol biopsies with i-SCAN or acetic acid chromoendoscopy, use of i-SCAN was comparable to acetic acid and superior to random biopsies in

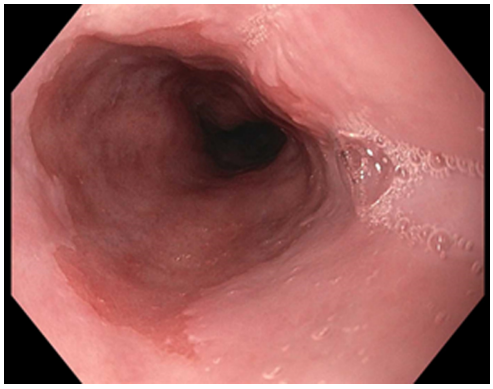

Figure 1 Barrett's esophagus segment under while light high definition endoscopy.

diagnosing intestinal metaplasia[9]. More data is needed to assess the right setting for tissue diagnosis for i-SCAN.

BLI or endoscopy (FUJIFILM ELUXEO 7000®) is a high quality optical technology that aims to provide enhanced visualization and differentiation of mucosal surfaces and vessel structures. BLI is felt to assist in better identifying changes in surface relief, defined as subtle elevations and depressions relative to normal surrounding flat mucosa. A 2018 study aimed to evaluate the additional value BLI could provide over WLE for identifying BE neoplasia[14]. Findings from this study showed that BLI added value to WLE for visualization of BE neoplasia and that experts appreciated BLI more than WLE for the delineation of BE neoplasia especially in lesions that were difficult to delineate with WLE alone[14].

Auto fluorescence imaging: Mucosa contains endogenous tissue fluorophores, which are biological structures that emit fluorescent light when exposed to light of a shorter wavelength. Auto flourescence imaging (AFI) operates on the principle that mucosa differs in the fluorescence it admits based on the type of tissue. For instance, while normal mucosa appears green under fluorescence excitation, dysplasia and neoplasia appears "magenta or purple"[9]. Using these principles, AFI can detect and characterize changes in mucosa.

Several early studies have shown AFI has good sensitivity increasing the detection of HGD and early neoplasia, however, specificity is poor with a high false positive rate[4,9]. Endoscopic trimodal imaging (ETMI) combines AFI with WLE and NBI in an attempt to maintain the sensitivity and reduce the false positive rate seen with AFI alone. Despite lowering the false positive rates, several studies have been unable to show a difference in detection rates between ETMI with targeted biopsies and standard endoscopy with random biopsies[9]. While AFI may be helpful as an adjunct to WLE, due to the high false positive rates, AFI alone is not an adequate replacement for current guideline recommendations.

Microscopic endoscopy: In conjunction with WLE and other advanced endoscopic imaging techniques, microscopic endoscopy allows for a real-time histological assessment of the esophageal mucosa during endoscopy[9].

Confocal laser endomicroscopy (CLE) is an advanced imaging technique that can magnify mucosa up to 1000 times to acquire submucosal images up to 250 micrometers below the mucosal surface. Most CLE studies have been performed using either endoscopic CLE (eCLE) where a confocal microscope is placed in to the tip of an endoscope or probe-based CLE (pCLE) where a probe can be introduced through an accessory channel. Given that premalignant lesions such as BE with dysplasia are challenging to identify with conventional screening, both eCLE and pCLE use a blue laser light and a fluorescent to enhance mucosal structures that are vascular-supplied[4,8-9,15] (Figure 4).

One approach to improve detection has been to develop a peptide to better find molecular changes. In particular, a fluorescently labeled peptide has been developed that specifically binds to HGD and EAC. In a 2013 study to evaluate the validity of this approach, after topically applying the peptide to the esophagus, confocal endomicroscopy was performed. In cases of esophageal neoplasia, the results showed a 3.8 fold greater fluorescent intensity compared to BE and normal squamous epithelium. The sensitivity of which was 75% and specificity was 97%. Additionally, the peptide is felt to be safe, with no toxicity in animal or patient studies[16].

Studies have shown an advantage in using CLE compared to WLE for detecting

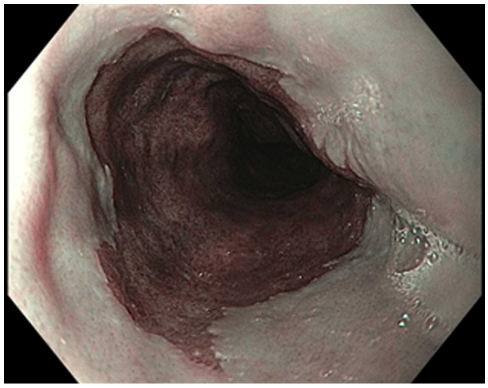

Figure 2 Barrett's esophagus using narrow band imaging.

HGD and EAC and reducing the number of biopsies required to make a diagnosis. Additionally, pCLE has a widely accepted classification criteria called the Miami criteria, reviewed in Table 5, that has been validated in random controlled trials[17]. One major concern regarding CLE is that all studies assessing its potential use were performed by expert endoscopists at "tertiary referral centers" where a higher percentage of patients with dysplasia are likely to be located[4]. Concerns over the practical use of CLE as a primary screening tool also exist due to high equipment costs, prolonged procedure time, and the training required using the equipment and interpreting images.

Endocytoscopy uses WLE and special magnification lenses to allow microscopic evaluation of the mucosa. Similar to dye-based chromoendoscopy, a contrast agent, usually methylene blue is applied to the surface of the mucosa, then depending on the system used, magnification can be up to 1400-fold of normal[9]. Studies have been performed to evaluate effectiveness in diagnosing squamous esophageal cancer and dysplasia and results have been variable. Currently, Endocytoscopy is not universally used for evaluation in patients with BE[9]. Overall, Endocytoscopy has shown promise in identifying dysplastic and neoplastic lesions with its primary limitation owing inability to perform wide-field screening of the mucosa. As such, one potential future application could include its use as an adjunct to other techniques to better visualize specific, targeted lesions[18].

Optical coherence tomography/volumetric laser endomicroscopy: Optical coherence tomography (OCT) is similar to ultrasound except that it uses light waves rather than sound waves to obtain subsurface, cross-sectional images of a mucosal surface. During standard endoscopy, images are obtained by introducing a catheter through the accessory channel[9,15]. One prospective clinical study assessing the presence of dysplasia in patients with BE using OCT found an 83% sensitivity and 75% specificity respectively. Several other studies have been performed to evaluate OCT and overall results have varied[9].

Optical frequency domain imaging otherwise referred to as volumetric laser endomicroscopy (VLE) is similar to OCT but allows for high resolution, high-speed acquisition of larger areas of the mucosal surface. In practice, VLE can be used to screen for BE, for surveillance of BE and for mapping prior to ablation or endoscopic resection similar to other advanced imaging technology. VLE also has the ability unlike other technology to evaluate for residual BE below neosquamous mucosa after endoscopic therapy[15]. Studies are now starting to focus on obtaining interobserver agreement regarding image interpretation and correlating images with histology[9].

Tethered capsule endomicroscopy: Tethered capsule endoscopy (TCE) is a new device that obtains images evaluating for BE by utilizing the imaging capabilities of OCT through the use of a tethered capsule. The pilot study performed in 2012 to test the overall safety and acceptability of the TCE device resulted in no adverse events and 89% of patients able to swallow the capsule. Additionally, of the patients tested, 62% recorded they would prefer TCE to endoscopy[19]. Another study using TCE evaluated 17 participants with suspicion or confirmed BE. Of the 17 patients, 13 had an esophagogastroduodenoscopy (EGD) within 12 mo of TCE and were able to swallow the capsule[19]. A blinded comparison of Prague C and M criteria for BE in TCE *vs* EGD was performed. Findings showed a strong to very strong correlation (r = 0.7-0.83, P < 0.5) for circumferential (C) extent and a strong correlation (r = 0.77-0.78, P < 0.01) for maximum (M) extent of BE[19].

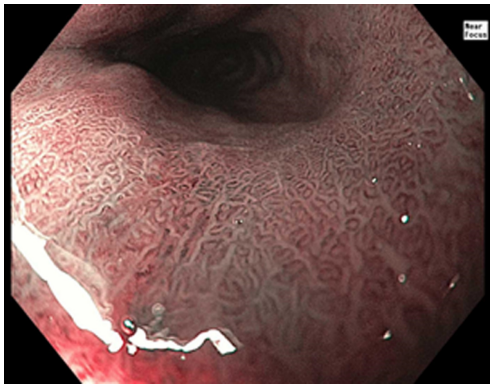

Figure 3 Barrett's esophagus using zoom magnification endoscopy (near focus).

Spectroscopy: Spectroscopy uses variation in scattered light across a full spectrum to obtain information on crowding, vascularity, size and tissue structure[9]. Raman spectroscopy specifically detects scattered light that has been changed in wavelength and creates characteristic peaks that correspond to normal vs abnormal mucosa. Early studies have shown good success in real-time detection of BE and neoplasia.

Other advanced technologies

Wide area transepithelial sampling with 3-dimensional tissue analysis: Wide area transepithelial sampling with computer 3-dimensional analysis (WATS-3D) is a new technique for screening and surveillance of BE. WATS-3D is able to obtain transepithelial specimens of BE by using a unique abrasive brushing instrument. The samples of tissue are then analyzed through a high-speed computer system to find the most suspicious cells which can then be reviewed by a pathologist[1,4,20].

In a multicenter prospective randomized trial that included 160 patients with BE, WATS-3D plus Seattle protocol was compared to Seattle protocol alone to determine if the combination protocol could improve the detection of dysplasia and neoplasia. In this study, Seattle protocol alone detected only 7 cases of HGD and neoplasia. With the addition of WATS-3D, an additional 23 cases of HGD and neoplasia were detected that were not found using Seattle protocol alone[1,4].

A second, larger prospective trial was performed that evaluated more than 4000 patients with suspected or established BE[1]. Patient either underwent EGD with Seattle protocol biopsies alone or Seattle protocol plus WATS-3D. In the group that underwent the protocol alone, BE was detected in 594 patients *vs* 799 patients tested by WATS-3D. Of the 799 patients diagnosed with BE by WATS-3D, 493 of these patients were not diagnosed with BE by Seattle protocol. Unique to this study was the evaluation for LGD. In the group tested with WATS-3D, 33 patients were diagnosed with LGD. Of these 33 patients, 23 had negative results for LGD by Seattle protocol alone[1]. Early results have been promising for the potential implementation of WATS-3D to improve efficiency for BE surveillance or possibly even screening however more research is required to determine its generalizability for wide-spread use.

Cytosponge™: Cytosponge™ (Medtronic, Menneapolis, MN, United States) is a novel device that consists of an ingestible gelatin capsule on a string. Once the device makes it to the stomach, the capsule dissolves and a small sponge is revealed that can then be withdrawn through the esophagus and out of the mouth by pulling the string. During this process, the sponge is able to collect esophageal cells to screen for different disease processes like BE dysplasia, and esophageal carcinoma. Once the cells are collected, the sponge is then tested to evaluate for trefoil factor 3 (TFF3) which is a biomarker for BE. Identification of this biomarker helps to distinguish BE from gastric cells and squamous cells within the esophagus[1].

Several prospective trials have been performed to evaluate the accuracy of the Cytosponge™ TFF3 test in screening for BE. The BE Screening Trial 1 (BEST1) cohort study looked at 501 patients with previous prescriptions for acid suppression[1]. Testing with Cytosponge™ with TFF3 showed 73% sensitivity and 94% specificity for patients with short segment BE which improved to 90% sensitivity and 93.5% specificity for long segment BE. The BE Screening Trial 2 (BEST2) subsequently evaluated 1110 patients with Cytosponge™ and endoscopy[1]. Findings from this trial yielded a sensitivity of 80% and specificity of 92% for short segment BE. Sensitivity increased to 87% in those with long segment BE[1].

In regards to safety, a multicenter review of 5 prospective trials using

Table 4 Barrett's international narrow band imaging group classification for Barrett's esophagus with narrow band imaging[3]

Mucosal pattern	
Circular, ridged/villous, or tubular	Regular
Absent or irregular	Irregular
Vascular pattern	
Blood vessels situated regularly along or between mucosal ridges and/or showing normal long branching patterns	Regular
Focally or diffusely distributed vessels not following normal architecture of the mucosa	Irregular

Cytosponge™ was published in August 2018[21]. Of 2672 Cytosponge™ procedures across these five trials, only two adverse events occurred related to the device. The adverse events included a single case of minor pharyngeal bleeding and a single case of device detachment. Additionally this study showed that patients tolerated the device well with > 90% achieving a successful swallow on the first attempt [21].

The Cytosponge™ offers the convenience of administration in addition to a cost effective alternative to traditional techniques. A recent study compared the cost-effectiveness of Cytosponge™ followed by endoscopic treatment to endoscopic screening followed by endoscopic treatment and found Cytosponge™ screening followed by endoscopic treatment to be more cost effective[22].

Transnasal endoscopy: Transnasal endoscopy (TNE) is a screening technique where a thin endoscope is introduced through the nares into the esophagus to evaluate for BE. TNE can be performed either in a hospital (hTNE) or mobile/outpatient (mTNE) setting and can be performed using only topical anesthetic without the need for sedation[1].

In a prospective study, 121 patients with either GERD symptoms or known BE were randomized to either transnasal endoscopy followed by standard endoscopy or standard endoscopy followed by transnasal endoscopy. The prevalence of BE in the two groups showed no significant difference at 26% and 30%, respectively (*P* value 0.503). Several other studies have demonstrated similar findings as well as better overall tolerance of transnasal endoscopy compared to standard endoscopy[1].

Similar to Cytosponge™, transnasal endoscopy is both easily tolerated and offers cost-effectiveness compared to standard endoscopy. In addition to reducing cost by eliminating the need for sedation, a new device called transnasal endosheath endoscopy (TNE-5000 with EndoSheath Technology, Vision Sciences, Inc., New York, NY, United States) utilizes a reusable endoscope with a disposable outer sterile sheath[1]. Overall, findings from studies involving transnasal endoscopy have shown promise as a potential future screening tool for BE.

Biomarker panels: Finding potential biomarkers for BE is a robust and exciting area of research. While several biomarkers for BE in the areas of dysplasia, genome markers, and gene expression alterations have been discovered, a single, ideal biomarker for BE has yet be identified[23].

One biomarker that has been proposed and shown promise as an adjunct to a traditional biopsy approach is immunostaining p53. This tumor suppressor gene becomes activated by injury to DNA to decrease cell multiplication to allow time for DNA repair and thus prevent damaged cells from replicating[24]. If the injury is too severe, then p53 can provoke cell death via apoptosis. One of the sentinel events that occurs in the progression of BE to neoplasia is the inactivation of p53. Given this occurrence, several studies are looking at p53 expression as a biomarker to determine risk for progression from BE to dysplasia and ultimately EAC[24]. Recently, a prospective study evaluated aberrant p53 expression to predict progression to HGD or EAC. Of 91 subjects with BE without dysplasia initially, 11 progressed to HGD or EAC. Aberrant p53 expression was evaluated in all of the subjects and was found significantly more often in those who developed HGD or EAC (63.6%) compared to subjects did not progress (7.5%)[25].

Another recent study assessed multiple proposed biomarkers in a case-control study to predict the progression of BE to EAC[26]. In this study, 130 patients with BE who progressed to HGD and/or EAC were compared with 130 patients with BE who never progressed. Using abnormal DNA, P53, Cyclin A, and *Aspergillus oryzae* lectin (AOL) in routine paraffin embedded biopsies sections, conditional logistic regression analysis was used on this patient population to estimate an odds ratio of progression. Findings from this study showed that of these biomarkers, expert LGD, AOL, and p53 all independently predicted progression of BE to neoplasia[26]. While research in this area is ongoing, early findings offer promise at identifying a tool to target more

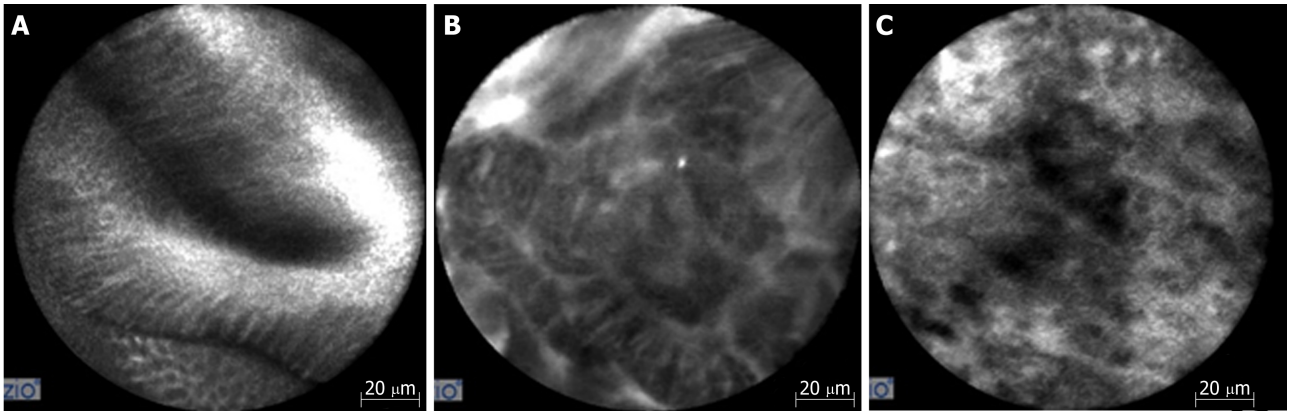

Figure 4 Confocal endomicroscopy imaging. A: Barrett's esophagus with intestinal metaplasia; B: Barrett's esophagus with high grade dysplasia; C: Esophageal adenocarcinoma.

aggressive surveillance and treatment strategies in patients with BE and potentially an improved method for screening in the future.

Breath testing using an electronic nose device: Electronic nose (e-nose) devices have been invented to utilize chemical to electrical interfaces to measure subtle differences in volatile organic compounds (VOCs). When combined with a machine-learning program, identification of these VOCs can be used as a noninvasive diagnostic test to differentiate certain disease states[27].

A recent cross-sectional study using this technology was performed on a group of 122 patients with a history of dysplastic BE to evaluate for the presence or absence of BE. Each subject provided a 5-min breathing sample in a fasting state prior to undergoing an upper endoscopy with biopsies. Using E-nose technology to categorize patients with findings characteristic of BE, detection of BE was found to have a sensitivity of 82% and specificity of 80%[27]. Future studies will be need to compare patients without BE but given its ease of use and portability, e-nose could be a potential screening tool for BE in the future.

CONCLUSION

In conclusion, as mentioned before, the incidence of EAC is rising. Given its poor prognosis, especially in the setting of having a known precursor lesion in BE that can be endoscopically monitored, identifying an efficient, cost-effective way to accurately screen for BE has become increasingly important. Research in this area is promising and primarily has focused on improved optical technology and advanced sampling techniques. The current techniques along with their advantages and disadvantages are listed below in Table 6. While promising in multiple areas, further research is required before a designated screening tool for BE can be universally implemented.

Table 5 Miami criteria for classifying Barrett's esophagus using confocal laser endoscopy[12]

Histology	Confocal characteristics
1 Normal Squamous Epithelium	Flat Cells with bright intrapapillary capillary loops
2 Non-dysplastic Barrett's Esophagus	Uniformed villiform architecture with dark goblet cells
3 Barrett's esophagus with high-grade dysplasia	Villiform structures with dark, irregular and thick borders
4 Adenocarcinoma	Disorganized villiform architecture and dilated irregular vessels

Table 6 Screening techniques for Barrett's esophagus[7]

	Advantage	Disadvantage
Standard definition white light endoscopy	Provides wide-field imaging and is widely available	Decreased sensitivity when compared to high definition
High definition white light endoscopy	Provides wide-field imaging and is widely available with improved image quality	Cost of procedure, sedation and in some cases updating entire endoscopy system. Some concerns over missed rates of dysplastic lesions
Dye-based chromoendoscopy	Provides wide-field imaging with benefit of mucosal enhancement	Additional steps in procedure are time consuming and some concerns over harm of contrast
Narrow band imaging	Provides wide-field imaging and is widely available with improved sensitivity and without need for contrast. Relatively cheap.	Still requires white light endoscopy as an adjunct with unclear evidence on its benefits when compared to white light endoscopy alone
Flexible intelligent chromoendoscopy and i-SCAN	Provides wide field imaging without the need for contrast	Not widely available and not enough research to determine benefits compared to standard of car
Blue light imaging	Helpful in defining subtle changes in elevation and depression of the mucosa	Beneficial as an adjunct to WLE only and hence requires similar costs. Not widely available.
Auto flourescence imaging	Provides wide field imaging with improved sensitivity and without the need for contrast	Poor specificity with high false positive rate.
Confocal laser endomicroscopy	Provides *in vivo* information, has a validated scoring classification, and can be used with any endoscope	Does not provide wide-field imaging, requires fluorescein prolonging procedure time, requires expert interpretation and expensive
Endocytoscopy	Increases ability to identify dysplastic and neoplastic lesions	Does not provide wide-field imaging and requires giving contrast agent
Optical coherence tomography	Provides *in vivo* information without need for contrast or fluorescein. Ability to evaluate subsurface	Does not provide wide-field imaging and research has varied and is ongoing
Volumetric laser endomicroscopy	Similar to OCT but provides high resolution, high speed images over wider surface area	Expensive and studies are still working to obtain interobserver agreement and correlating images with histology
Tethered capsule endomicroscopy	Utilizes same technology used for OCT and is safe, well tolerated by patients	Early in stages of research
Spectroscopy	Early studies have shown good success in real time detection of BE and neoplasia	Early in stages of research
wide area transepithelial sampling	Provides wide area sampling of tissue with high sensitivity and specificity and easy to use	Not yet widely available? Regarding cost and more research needed
Cytosponge	Generally safe and well tolerated with low cost	Still requires endoscopy for treatment if abnormality is identified
Transnasal Endoscopy	Generally safe and well tolerated with relatively lower cost than endoscopy without the need for general sedation. Can be used in clinic as well as hospital	While early studies have shown equivocal ability to diagnosis BE compared to conventional endoscopy, more research required
Biomarker panels	Early studies have shown ability to predict progression of BE from non-dysplastic to neoplasia	A single, ideal biomarker has not been delineated and more research is required.
Breath testing with an electronic nose device	Safe and well tolerated and easy to use with overall cost-effectiveness	Sensitivity and specificity are good but not great compared to some other methods and research at this point is limited

WLE: White light endoscopy; OCT: Optical coherence tomography; BE: Barrett's esophagus.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 May 7; 25(17): 2058-2070

DOI: 10.3748/wjg.v25.i17.2058

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Proton pump inhibitor: The dual role in gastric cancer

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Author contributions: Joo MK, Park JJ and Chun HJ performed research; Joo MK wrote the paper; Park JJ and Chun HJ revised the manuscript for critical intellectual contents.

Supported by Basic Science Research Program through the National Research Foundation of Korea, No. NRF-2016R1D1A1A02936964.

Conflict-of-interest statement: None of the authors have potential conflicts (financial, professional, or personal) that are relevant to this publication.

Manuscript source: Invited manuscript

Received: February 20, 2019
Peer-review started: February 20, 2019
First decision: March 5, 2019
Revised: March 17, 2019

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Abstract

Proton pump inhibitors (PPIs) are one of the most frequently used medications for upper gastrointestinal diseases. However, a number of physicians have raised concern about the serious side effects of long-term use of PPIs, including the development of gastric cancer. Recent epidemiological studies have reported a significant association between long-term PPI intake and the risk of gastric cancer, even after successful *Helicobacter pylori* eradication. However, the effects of PPIs on the development of pre-malignant conditions such as atrophic gastritis or intestinal metaplasia are not fully known, suggesting the need for comprehensive and confirmative studies are needed in the future. Meanwhile, several experimental studies have demonstrated the effects of PPIs in reducing chemoresistance in gastric cancer cells by modulating the acidic microenvironment, cancer stemness and signal transducer and activator of transcription 3 (STAT3) signaling pathway. The inhibitory effects of PPIs on STAT3 activity may overcome drug resistance and enhance the efficacy of conventional or targeted chemotherapeutic agents. Taken together, PPIs may "play dual role" in gastric carcinogenesis and treatment of gastric cancer.

Key words: Proton pump inhibitor; Gastric cancer; Drug resistance; Signal transducer and activator of transcription 3

Core tip: Recent epidemiological studies have demonstrated a significant increase in gastric cancer risk following the long-term use of proton pump inhibitors (PPIs). However, observational studies have fundamental limitations. PPIs may affect gastric cancer cells and the microenvironment by modifying the acidic conditions and inhibiting the cancer stemness *via* various signaling pathways including signal transducer and

Accepted: March 24, 2019
Article in press: March 25, 2019
Published online: May 7, 2019

P-Reviewer: Chiu CC, Link A, Mastoraki A, Tan HJ
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

activator of transcription 3, which in turn, reduces drug resistance to chemotherapy. In this review, we briefly summarize the current clinical outcomes of the effects of long-term PPI use and the development of gastric cancer, as well as experimental studies showing enhanced chemosensitivity in gastric cancer.

Citation: Joo MK, Park JJ, Chun HJ. Proton pump inhibitor: The dual role in gastric cancer. *World J Gastroenterol* 2019; 25(17): 2058-2070
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2058.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2058

INTRODUCTION

Gastric cancer is one of the most frequently found malignant solid tumors worldwide, and is the third leading cancer-related cause of mortality[1]. Advances in technical and clinical knowledge have increased the early detection of gastric cancer for prompt intervention and successful management[2]. However, a significant number of gastric cancer cases are still diagnosed in advanced stages with distant metastasis, resulting in poor prognosis. A recent pivotal prospective randomized study showed that the overall survival in gastric cancer with a single metastasis was not significantly different between patients receiving chemotherapy alone and patients treated with gastrectomy combined with chemotherapy. The study also reported that the median overall survival was less than 18 mo[3].

Significant risk factors for gastric cancer include male gender; old age; ethnicity; *Helicobacter pylori* (*H. pylori*) infection; dietary factors, such as smoked food, high salt intake, pickled vegetables and nitrated meat; smoking; and family history. In terms of gastric factors, atrophic gastritis and intestinal metaplasia are proven pre-cancerous conditions[4]. A South Korean study showed that the risk of developing gastric cancer was increased more than 10 fold among subjects who had intestinal metaplasia compared with subjects who did not[5]. Thus, avoidance and optimal surveillance of risk factors are mandatory for the prevention of gastric cancer.

Proton pump inhibitors (PPIs) are the most potent acid inhibitors ever developed: they act by blocking the H^+/K^+ ATPase of parietal cells[6]. PPIs are powerful acid inhibitors and there, are widely used as drugs of choice for the treatment of gastroesophageal reflux disease (GERD) and drug-induced peptic ulcers. Long-term use of PPIs may facilitate the optimal management of GERD combined with severe complications such as esophageal stricture[7], and in practice, the long-term prescription of a PPI is often preferred as maintenance therapy, even for uncomplicated GERD patients[7]. However, there are rising concerns about the potential side effects of long-term PPI intake which include *Clostridium difficile* infection, pneumonia, bone fractures, dementia, chronic renal disease and small intestinal bacterial overgrowth[8]. Recent observational studies demonstrated a positive association between PPI use and malignant or pre-malignant tumors of the gastrointestinal tract. Reflecting recent trends, a recent expert opinion suggests that the dose of long-term PPIs should be periodically re-evaluated and that the lowest possible effective dose needs to be prescribed[9]. However, several experimental studies showed significant anti-tumor effects of PPI in cancer cells such as Barrett's adenocarcinoma and melanoma cells[10,11], and suggested that PPIs may contribute to reducing of tumor resistance to chemotherapy[12]. Several experimental studies have demonstrated this "unexpected" effect of PPIs in gastric cancer cells. In this review, we focused on the dual action of PPIs in gastric cancer. We not only summarized the clinical outcomes correlating the development of gastric malignancy with long-term use of PPI but presented an experimental hypothesis and experimental evidence supporting the anti-tumorigenic, drug-sensitizing effects of PPI in gastric cancer cells.

PPI AND GASTRIC CARCINOGENESIS

Hypothesis for causality of PPI and gastric cancer

The most plausible hypothesis for the association between long-term PPI intake and the development of gastric cancer is mediated *via* hypergastrinemia due to the reduced secretion of gastric acid[13]. This reduced acidity, in turn, triggers a proliferation of enterochromaffin-like cells (ECL cells), which express gastric

cholecystokinin-2 (CCK-2) receptors and are the target cells of gastrin in the oxyntic mucosa, and formation of neuroendocrine tumors (NETs)[14]. The somatostatin-mediated negative feedback of gastrin release on antral G-cells is frequently inhibited by gastric hypochlorhydria caused by long-term PPI use and other anti-acidic drugs, which leads to hypergastrinemia and hyperplasia of the gastric mucosa or ECL-cells[15]. The second hypothesis is that gastrin *per se* has a trophic effect on the oxyntic mucosa, as well as on ECL cells, under hypergastrinemic conditions such as chronic atrophic gastritis or prolonged PPI use[16]. A previous animal study showed that a high salt diet administered to *H. pylori*-infected Mongolian gerbils significantly increased serum gastrin levels and mucosal inflammation, which were ameliorated by a gastrin antagonist[17]. A recent case-control study showed that the subgroup with the highest quartile of serum gastrin levels was significantly associated with gastric non-cardia adenocarcinoma [fully adjusted odds ratio (OR) = 1.92; 95% confidence interval (CI): 1.21-3.05], as well as NET (age-adjusted continuous model OR = 4.67; 95%CI: 2.67-8.15)[18].

However, a molecular link between ECL cell hyperplasia and gastric adenocarcinoma is less relevant than gastric NET in general[14]. Nevertheless, a fraction of gastric adenocarcinomas originates from ECL cells. A previous study using human gastric carcinoma tissues showed that ECL cell markers, such as chromogranin A, synaptophysin, histidine decarboxylase and neuron specific enolase, were predominantly expressed in diffuse type gastric cancer rather than intestinal type gastric cancer[19]. Moreover, several pathologic studies have shown that most periodic acid-Schiff (PAS)-positive signet ring cell carcinomas abundantly expressed ECL-cell markers, but not mucin, suggesting that signet ring cell carcinoma might be a consequence of dedifferentiation from ECL cells toward signet ring cells with PAS-positive cytoplasm[20,21]. At the present stage, the effect of PPIs might be summarized by the following statement. PPIs reduce gastric acid secretion and lead to hypergastrinemia with the proliferation of ECL cells in the oxyntic gland, partially and theoretically explaining the potential association between PPI and gastric cancer, or at least, the enhancement of *H. pylori*-associated gastric carcinogenesis[22] (Figure 1). However, this hypothesis is often insufficient to elucidate the mechanism of PPI-induced gastric carcinogenesis. Moreover, a recent pivotal translational study demonstrated that PPI-treated patients showed similar microbial diversity compared with normal subjects while patients with *H. pylori*-induced atrophic gastritis manifested a lower bacterial abundance and diversity. This finding suggested that PPIs do not significantly alter gastric microbiota nor do they contribute significantly to the development of gastric cancer[23].

Clinical evidence supporting the association of PPI and development of gastric cancer

Previously, three retrospective, case-control studies from databases of Western countries analyzed the increased risk of gastric cancer with PPI intake[24-26]. These studies included relatively small number of gastric cancer cases (approximately 2000) and missed several major confounding factors, such as *H. pylori* infection status, dietary patterns or family history of gastric cancer. A meta-analysis which included the above three case-control studies, showed that the pooled relative risk (RR) of gastric cancer following PPI use was 1.43 (95%CI: 1.23-1.66) using both fixed- and random-effects models. However, the subgroup analysis failed to show a dose-dependent relationship between PPI and gastric cancer (PPI < 12 mo: pooled RR = 1.73, 95%CI: 1.24-2.52; > 12 mo: pooled RR = 1.42, 95%CI: 0.98-2.07; > 36 mo: pooled RR = 2.45, 95%CI: 1.41-4.25). The authors stated that colonization with *H. pylori* and adequate long-term use of PPI synergistically increased the risk of gastric cancer[27]. Another previous meta-analysis showed a similar effect of acid suppressive drugs on gastric cancer (adjusted OR = 1.42; 95%CI: 1.29-1.56); however, the pooled effect was confounded by H2RA, and was not solely due to PPI[28].

Recently, Cheung *et al*[29] showed a positive correlation between PPI and gastric cancer in *H. pylori*- infected patients who underwent eradication therapy. In this large-scale, population-based study involving a Hong Kong health database, the authors enrolled more than 63000 adult patients who were prescribed with a clarithromycin-based triple therapy. Current *H. pylori* infection was diagnosed by an invasive or non-invasive study. To eliminate protopathic bias, patients who were diagnosed with gastric cancer within six months before the study or within 12 mo after *H. pylori* eradication therapy were excluded[30]. Furthermore, to minimize the effect of *H. pylori*-induced gastric carcinogenesis, only patients successfully treated with eradication therapy were enrolled. Failure of *H. pylori* eradication was therapy identified if patients were prescribed subsequent medication of (1) repeated standard triple therapy, (2) bismuth-containing second-line quadruple therapy, or (3) rifabutin-based third-line therapy. During a median follow-up of 7.6 years, 153 patients (0.24%)

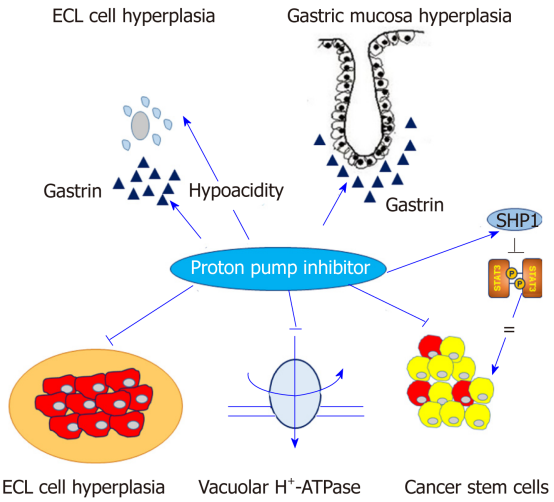

Figure 1 Contrast effects of proton pump inhibitors in normal gastric mucosa and gastric cancer cells. Proton pump inhibitors (PPIs) induce hypergastrinemia and hypochlorhydria, which may contribute to enterochromaffin-like cell hyperplasia and proliferation of gastric mucosa. Conversely, PPIs may modify the acidic tumor microenvironment and inhibit vacuolar H^+-ATPase or signal transducer and activator of transcription 3 activity in gastric cancer cells. Arrow indicates the positive effect and straight line indicates the inhibitory effect. ECL: Enterochromaffin-like; SHP1: Src homology 2 domain-containing protein tyrosine phosphatase 1; STAT3: Signal transducer and activator of transcription 3.

developed gastric cancer. PPI use significantly increased the risk of gastric cancer [hazard ratio (HR) = 2.44; 95%CI: 1.42-4.20], unlike H2RA (HR = 0.72; 95%CI: 0.48-1.07). Moreover, the positive association between PPI and gastric cancer showed dose- and duration-dependent relationship[29]. This study was significant in that it demonstrated the increased risk of gastric cancer with long-term use of PPIs, even after successful eradication of *H. pylori*. However, it had several important limitations. First, due to the fundamental limitations of observational studies, several baseline characteristics such as age, metabolic diseases (diabetes, hypertension, and dyslipidemia) and other major comorbidities (ischemic heart disease, stroke, congestive heart failure, and chronic renal failure) were significantly biased between the case and control groups. Consequently, gastric atrophy, salty food intake or obesity, which are related to gastric cancer development, may have occurred more frequently in the PPI user group, even after statistically sophisticated propensity-score matching[31]. Second, important confounding factors of gastric cancer such as gastric atrophy, intestinal metaplasia and dietary patterns were excluded[32]. Third, the authors determined success or failure of *H. pylori* eradication only based on prescription histories. Thus, a portion of the enrolled patients may have continued to harbor *H. pylori* infection, even after eradication, and the carcinogenic effect of *H. pylori* may not have been completely eliminated.

A Swedish nationwide population-based cohort study recruited almost 800000 Swedish adults who were undergoing maintenance therapy with PPIs, and the significance incidence ratio (SIR) of gastric cancer was 3.38 (95%CI: 3.23-3.53), which was consistent regardless of gender, age, indications for PPIs (*i.e.*, GERD), concomitant use of anti-inflammatory drugs such as aspirin or nonsteroidal anti-inflammatory drugs (NSAIDs) and the subsite of gastric cancer (cardia and non-cardia cancer)[33]. In this study, the authors restricted enrollment to subjects who were exposed to PPI maintenance therapy, defined as a cumulative defined daily dose of at least 180 d during the study period, to reduce the possibility of reverse causality of PPI and gastric cancer. However, this study also failed to establish a causal relationship between gastric cancer and long-term use of PPI, in that the SIR of gastric cancer did not show any duration-dependent pattern. Furthermore, crucial information such as the current *H. pylori* infection status was missing. Clinical studies correlating the long-term use of PPIs with gastric cancer are summarized in Table 1.

Interestingly, clinical outcomes supporting the effect of long-term PPI use on the development of pre-cancerous conditions, such as atrophic gastritis or intestinal metaplasia, are lacking. A previous cohort study showed that 30% (18/59) of patients who were treated with long-term omeprazole and *H. pylori* infection at baseline developed atrophic gastritis, which was significantly higher than non-omeprazole group[34]. Meanwhile, previous randomized controlled trials (RCTs) showed that the proportion of patients who progressed to gastric corpus glandular atrophy and

Table 1 Summary of clinical studies associating gastric cancer with long-term use of proton pump inhibitors

Author, year	Study design, country	Study period	Source of database	No. of case and control	Information of PPI	Adjustment	Main outcomes
Garcia-Rodriguez *et al*[24], 2006	Nested case-control, retrospective, United Kingdom	1994-2001	The general practitioners research database in the United Kingdom	522/10000	Duration, indication	Age, sex, calendar year, smoking, alcohol consumption, body mass index, gastro-esophageal reflux, hiatal hernia, peptic ulcer, and dyspepsia	OR for gastric cardia adenocarcinoma: 1.06 (0.57-2.00); gastric non-cardia adenocarcinoma: 1.75 (1.10-2.79)
Tamim *et al*[25], 2008	Case control, retrospective, Canada	1995-2003	Quebec health insurance plan	1598/12991	Type, dose, exposure time	Number of drug prescriptions, total length of hospitalizations, number of visits to GPs, specialists, and emergency rooms during the year before the diagnosis	Adjusted OR: 1.40 (1.08-1.51); 1st quartile: 1.66 (1.24-2.23); 2nd quartile: 1.37 (1.00-1.88); 3rd quartile: 1.57 (1.17-2.10); 4th quartile: 1.20 (0.85-1.70)
Poulsen *et al*[26], 2009	Population-based cohort, retrospective, Denmark	1990-2003	Danish National Health-care System	109/not reported	Type, year of follow-up, no. of prescription	Age, gender, calendar period, gastroscopy (≥ 1 yr before censoring events), use of NSAIDs and *H. pylori* eradication	IRR for gastric cancer: 1.2 (0.8-2.0) among PPI users with the largest number of prescriptions (15+) or the longest follow-up (5+)
Cheung *et al*[29], 2018	Population-based cohort study, retrospective, Hong Kong	2003-2012	Clinical Data Analysis and Reporting System of the Hong Kong Hospital Authority	153/63397	Frequency, duration	Age of receiving *H. pylori* eradication therapy, sex, smoking, alcohol use, comorbidities, concomitant medications	HR for gastric cancer: 2.44 (1.42-4.20); ≥ 1 yr: 5.04 (1.23-20.61); ≥ 2 yr: 6.65 (1.62-27.26); ≥ 3 yr: 8.34 (2.02-34.41). The adjusted absolute risk difference for PPIs *vs* nonPPIs use: 4.29 (1.25-9.54) per 10000 person-yr.
Brusselaers *et al*[33], 2017	Population-based cohort study, retrospective, Sweden	2005-2012	The Swedish Prescribed Drug Registry	2219/794848	Indication, cumulative defined daily dosages, estimated number of days	Age, sex, calendar period, indication of PPI, maintenance use (≥ 180 d) of aspirin or other NSAIDs	SIR: 3.38 (3.23-3.53) in both sexes, all age groups and all indication groups; < 1 yr: 12.82 (12.19-13.47); 1.0-2.9 yr: 2.19 (1.98 to 2.42); 3.0-4.9 yr: 1.10 (0.91-1.31); ≥ 2 yr: 0.61 (0.52-0.72)

PPI: Proton pump inhibitor; OR: Odds ratio; GP: General physician; NSAID: Nonsteroidal anti-inflammatory drug; *H. pylori*: *Helicobacter pylori*; IRR: Incidence rate ratio; HR: Hazard ratio; SIR: Standardized incidence ratio.

intestinal metaplasia was not significantly different between the long-term omeprazole-treated group and the control group[35,36]. A previous study based on histopathologic evaluation of gastric biopsy samples showed that only a small number of patients had worsening of their gastritis score for gastric atrophy and intestinal metaplasia following 12 mo of esomeprazole therapy: 1.4% had atrophy and 0.5% had intestinal metaplasia on the antrum, and 1.2% had atrophy and 0.8% had intestinal metaplasia on the corpus[37]. Recently, the Cochrane Database systematically reviewed four RCTs for the effects of long-term PPI intake on corporal atrophy and intestinal metaplasia. The meta-analysis showed that OR for corporal atrophy was

1.50 (95%CI: 0.59-3.80; P = 0.39), and the OR for intestinal metaplasia was 1.46 (95%CI: 0.43-5.03; P = 0.55), both of which failed to reach statistical significance[38]. Clinical studies associating the long-term use of PPIs with pre-malignant conditions of gastric cancer are summarized in Table 2.

In summary, several studies have shown a significant relationship between long-term PPI use and the risk of gastric cancer. However, the evidence is far from definitive because of limitations of research design and omission of several major confounding variables. Furthermore, conflicting data also exist. For example, although United States is one of the countries with the most frequent and long-term use of PPI, the incidence of gastric cancer is relatively low[39]. Thus, robust evidence including well-designed, large-scale prospective studies are needed to support the potential association between long-term PPI use and gastric cancer

PARADOXICAL ACTION OF PPI IN GASTRIC CANCER CELLS

Effects of PPI on tumor resistance

The unexpected effects of PPIs on solid tumors, including gastric cancer occur by several potential mechanisms (Figure 1).

First, a change of acidity occurs in the tumor microenvironment, for instance in solid tumors, the extracellular pH is acidic and the intracellular pH is neutral-to-alkaline, whereas the pH of the microenvironment in normal tissue usually remains alkaline[40]. This phenomenon leads to decreased intracellular concentrations of cytotoxic drugs that are weakly basic, such as cisplatin, 5-fluorouracil, vinblastine or doxorubicin[12]. PPIs contribute to overcoming drug resistance and enhance chemosensitivity by inhibiting the vacuolar H^+-ATPase (V-H^+-ATPase) of tumor cells, alkalizing the tumor microenvironment and retaining weakly basic cytotoxic drugs within the intracellular targets[11]. An *in vitro* study showed that pretreatment with omeprazole and esomeprazole significantly increased the sensitivity of cytotoxic drugs, such as cisplatin, 5-fluorouracil and vinblastine in various solid cancer cell lines with multi-drug resistance phenotypes[41].

Second, the modulation of cancer stemness plays a role. Cancer stem cells (CSCs) play a key role in the development of chemoresistance as well as cancer metastasis[42]. Several family proteins of ATP binding cassette (ABC) transporters such as P-glycoprotein, multi-drug resistance (MDR) associated protein-1 (MRP-1), lung resistance protein (LRP) and breast cancer resistance protein (BCRP) are highly expressed in CSCs and contribute to MDR by enhancing the activity of drug efflux pumps[43]. PPIs reduce chemoresistance *via* modification of anaerobic glycolysis and ABC transporters in solid cancer cells[44].

Several experimental studies have demonstrated the anti-tumor effects and the ability of PPIs to overcome MDR in gastric cancer. An *in vitro* and *in vivo* study showed that pantoprazole treatment selectively induced apoptotic cell death in gastric cancer cells, while normal gastric epithelial cells were resistant to pantoprazole[45]. Pretreatment of PPIs effectively inhibited the activity of V-H^+-ATPase, which resulted in an increased concentration of cytotoxic drugs in gastric cancer cells[46]. Several *in vitro* studies demonstrated putative downstream effectors following the inhibition of V-H^+-ATPase by PPIs in gastric cancer cells, such as the dephosphorylation of LRP6 and the inhibition of Wnt/β-catenin signaling[47] or PI3K/Akt/mTOR/HIF-1α signaling pathways[48]. A study showed that high-dose esomeprazole inhibited the release of exosomes and exosome-related micro-RNAs such as miR-494-3p, miR-6126 and miR-3934-5p, which are closely associated with tumor invasion, metastasis, adhesion and migration, and in turn, regulated the HIF-1α-FOXO1 axis to induce apoptosis and inhibit cellular migration and invasion in gastric cancer cells[49]. In summary, PPIs modulate the acidic microenvironment, and regulate V-H^+-ATPase and cancer stemness of various cancer cells including gastric cancer, and contribute to the reduction of tumor resistance to chemotherapeutic agents.

PPI modulating SHP-1/STAT3 signaling axis

It is well known that signal transducer and activator of transcription 3 (STAT3) signaling pathway plays a pivotal role in the invasion of gastric cancer[50]. In brief, phosphorylated STAT3 forms a homodimer for nuclear translocation, where it acts as a transcription factor to activate various target genes including cellular migration and invasion in epithelial cells. It also activates surrounding immune cells to regulate various immunologic reactions favoring cancer cell survival, such as the production of inflammatory cytokines and formation of pre-metastatic niches[51]. Various *in vitro* and *in vivo* studies have demonstrated that fully activated STAT3 induced epithelial-mesenchymal transition (EMT) *via* upregulation of relevant target genes such as

Table 2 Summary of clinical studies associating of gastric pre-malignant conditions with long-term use of proton pump inhibitors

Author, year	Study design, country	Source of database	No. of PPI and control group	Information of PPI	Aims	Main outcomes
Kuipers *et al*[34], 1996	Prospective cohort, Netherland/ Sweden	Reflux esophagitis cohort (fundoplication/ omeprazole)	105 (PPI)/ 72 (fundoplication)	Type (omeprazole only), dose (20 and 40mg), duration (5 years)	Corpus gastritis, atrophic gastritis	Atrophic gastritis: 0/31 (fundoplication group) *vs* 18/59 (omeprazole group) with *H. pylori* infection at baseline ($P < 0.001$); 0/41 (fundoplication group) *vs* 2/46 (omeprazole group) without *H. pylori* infection at baseline ($P = 0.62$)
Lundell *et al*[35], 1999	RCT, Sweden	RCT comparing the efficacy of omeprazole and ARS	155 (PPI)/155 (ARS)	Type (omeprazole only), duration (3 years)	Gastric corpus glandular atrophy, intestinal metaplasia of corpus mucosa	No difference in glandular atrophy between *H. pylori*-infected omeprazole and ARS group ($P = 0.57$); No difference in intestinal metaplasia between *H. pylori*-infected omeprazole and ARS group.
Lundell *et al*[36], 2006	RCT, Sweden	RCT comparing the efficacy of omeprazole and ARS	117 (PPI)/98 (surgical arm)	Type (omeprazole only), duration (7 years)	Gastric corpus glandular atrophy	No significant change of gastric atrophy between *H. pylori*-negative omeprazole and ARS group; Two patients developed severe atrophy from none at baseline in *H. pylori*-infected omeprazole group, three patients developed mild atrophy from none at baseline in *H. pylori*-infected ARS group, no statistical difference.
Gental *et al*[37], 2003	Two RCTs, United States	Maintenance trial ($n = 519$), Safety trial ($n = 807$)	Maintenance trial: 519 (PPI)/169 (placebo); Safety trial: 807/PPI	Type (esomeprazole only), duration (6 months: maintenance trial; 12 months: safety trial)	Atrophy (antrum and corpus), intestinal metaplasia (antrum and corpus)	In the maintenance studies, the majority of omeprazole group had no change in the extent of atrophy and intestinal metaplasia. In the safety study, > 98% of omeprazole had either no change or improved atrophy scores in antrum and corpus, and intestinal metaplasia scores remained unchanged or improved compared with those that worsened.

PPI: Proton pump inhibitor; RCT: Randomized controlled trial; ARS: Anti-reflux surgery; *H. pylori*: *Helicobacter pylori*.

vimentin and survivin in gastric cancer cells[52-54]. Furthermore, clinical outcomes also showed that high level of phosphorylated STAT3 were significantly associated with regional lymph node metastasis and poor prognosis in gastric cancer patients[55-57]. STAT3 also plays as a key role in the activation of CSCs. A previous study showed that gastric cancer-derived mesenchymal stem cells (GC-MSCs) secreted interleukin (IL)-6 and activated STAT3 in neutrophils. These GC-MSCs-primed neutrophils

induced transdifferentiation of normal MSCs to cancer associated fibroblasts[58]. Thus, STAT3 may present a primary target for the inhibition of gastric cancer invasion.

Several inhibitors of STAT3 including direct STAT3 inhibitors or inhibitors of upstream kinases, such as janus kinase 2 (JAK2) or Src kinase have been introduced and evaluated in experimental studies[59-61]. However, clinical studies involving gastric cancer patients are lacking, and technical limitations due to large surface of the target area have demonstrated the need for more stable and effective direct STAT3 inhibitors[62]. Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1), a non-receptor type protein Tyr phosphatase (PTPase), has attracted attention as an effective inhibitor of STAT3 activity[63]. SHP-1 acts as a protein Tyr PTPase and induces the dephosphorylation of STAT3 in various cell types. It is abundantly expressed and has been mostly evaluated in cells of hematopoietic lineage, such as macrophages, neutrophils, monocytes and mast cells[64]. Pivotal studies demonstrated that the expression of SHP-1 was aberrantly reduced by CpG island hypermethylation in lymphoma and leukemia[65,66]. Recently, the suppressive effect of STAT3 by SHP-1 has been evaluated in solid tumors. Chen *et al*. showed that several multiple kinase inhibitors such as sorafenib, dovitinib and regorafenib effectively induced SHP-1 in hepatocellular carcinoma, and in turn, suppressed STAT3 activity *via* dephosphorylation[67-69]. The function of SHP-1 has been recently evaluated in gastric cancer. Sun *et al*. showed that the expression of SHP-1 was the highest in normal gastric epithelium, followed by intestinal metaplasia and dysplasia and was the lowest in gastric cancer tissues[70], SHP-1 combines with a transmembrane protein with epidermal growth factor and two follistatin motifs 2 (TMEFF2) to inhibit STAT3 phosphorylation in gastric cancer cells and *H. pylori*-infected gastric epithelial cells[71].

We also previously showed that the expression of SHP-1 was aberrantly reduced following CpG island hypermethylation in various gastric cancer cell lines, and enhanced expression of SHP-1 in gastric cancer cells effectively dephosphorylated STAT3, resulting in downregulation of various target genes involved in cellular migration and invasion[72]. An *in vitro* study reported that PPIs exhibited a dose-dependent cytotoxicity and enhanced the sensitivity of cisplatin *via* inhibition of IL-6-stimulated STAT3 activity and its target genes[73]. Recently, we demonstrated that pantoprazole, a well-known PPI, effectively induced SHP-1 and downregulated phosphorylated-STAT3 levels in gastric cancer cells in a dose-dependent manner and modulated EMT markers[74]. Thus, we suggest that PPIs may act as effective STAT3 inhibitors *via* induction of SHP-1 in gastric cancer cells and play a role in the inhibition of progression of gastric cancer.

Application of PPIs in overcoming chemoresistance

Previous studies have demonstrated that the constitutive expression of STAT3 in gastric cancer was closely associated with the MDR of chemotherapeutic agents *via* enhanced expression of various oncogenes and downregulation of apoptotic genes[75]. Enhanced STAT3 activity also induced V-H^+-ATPase in gastric cancer cells, which abrogated the uptake of chemotherapeutic agents and contributed to the development of chemoresistance, as mentioned above[76]. Furthermore, recent studies showed that STAT3 activation reduced the efficacy of trastuzumab, a promising therapeutic antibody targeting HER2, *via* upregulation of MUC1 and MUC4[77], or the positive feedback loop of IL-6/STAT3/Jagged-1/Notch[78]. Thus, effective inhibition of STAT3 activity is considered the mainstay of intervention to overcome chemoresistance and effective management of advanced gastric cancer patients. A previous study demonstrated that pantoprazole effectively inhibited invasion and EMT of adriamycin-resistant gastric cancer cells *via* suppression of the Akt/GSK-β/β-catenin signaling pathway[79]. We recently found that a minimal dose of pantoprazole combined with docetaxel significantly induced SHP-1 expression, downregulated phosphorylation of STAT3, modulated EMT markers, and inhibited cellular migration and invasion in gastric cancer cells. Injection of both pantoprazole and docetaxel into nude mice significantly reduced the tumor volume of xenograft tumors of gastric cancer cells, compared with single administration of each drug[80]. Taken together, we suggest that a combination of PPIs during chemotherapy may play a role in enhancing the sensitivity and efficacy of chemotherapeutic agents including trastuzumab. Experimental studies reporting the effects of PPIs in gastric cancer cells and chemotherapeutic agents are summarized in Table 3. However, the lack of human studies and limited clinical relevance represent challenges that need to be addressed before PPIs are used to increase the effectiveness of chemotherapy for actual gastric cancer and improve patient prognosis. Further pre-clinical and clinical studies that are relevant to this hypothesis are needed.

Table 3 Summary of experimental studies investigating the effects of proton pump inhibitors in gastric cancer cells

Author, year	Study design	Type of PPI	Cell type	Main outcomes	Underlying hypothesis
Yeo *et al*[45], 2004	*In vitro, in vivo*	Pantoprazole	MKN 45, MKN 28, AGS, SNU 601, RGM-1 (normal gastric mucosa cell)	Apoptotic cell death in gastric cancer cells, but not in normal gastric mucosal cells, induced by pantoprazole	Modulation of heat-shock proteins (HSP 70, HSP 27)
Chen *et al*[46], 2009	*In vitro*	Pantoprazole	SGC7901	Inhibition of V-H^+-ATPase expression in a dose-dependent manner; enhancement of efficacy of anti-tumor drug (cisplatin) and increased apoptosis rate	Change of pH gradient (decrease of intracellular pH and reverse of the transmembrane pH gradient)
Shen *et al*[47], 2013	*In vitro*	Pantoprazole	SGC7901	Anti-proliferation, anti-invasive and pro-apoptotic effects, decrease of V-H^+-ATPase expression	Inhibition of LRP6 in Wnt/β-catenin signaling
Chen *et al*[48], 2018	*In vitro, in vivo*	Pantoprazole	SGC7901, SGC7901/MDR	Inhibition of V-H^+-ATPase expression in, SGC7901/MDR cells	Inhibition of P-gp and MRP1, and downregulation of PI3K/Akt/mTOR/HIF-1α signaling pathway
Guan *et al*[49], 2017	*In vitro, in vivo*	Esomeprazole	SGC7901	Enhancement of efficacy of anti-tumor drugs (cisplatin, paclitaxel, 5-FU); Inhibition of transformation of CAF	Regulation of HIF-1α-FOXO1 axis and inhibition of release of exosome and exosome-related microRNAs (tumor invasion, metastasis and TGF-beta pathway)
Huang *et al*[73], 2013	*In vitro*	Pantoprazole	SGC7901, GBC823, AGS	Inhibition of cellular proliferation and increase in the number of apoptotic cells	Inhibition of STAT3
Koh *et al*[74], 2018	*In vitro, in vivo*	Pantoprazole	AGS, MKN-28	Inhibition of cellular invasion, migration and modulation of EMT markers	Induction of SHP-1 and inhibition of JAK2/STAT3
Zhang *et al*[79], 2015	*In vitro*	Pantoprazole	Adriamycin-resistant SGC7901 (SGC7901/ADR)	Inhibition of cellular migration/invasion and modulation of EMT markers in SGC7901/ADR cells	Inhibition of Akt/GSK-β/βcatenin signaling
Joo *et al*[80], 2018	*In vitro, in vivo*	Pantoprazole	AGS	Enhanced cellular migration/invasion and anti-tumor effect of docetaxel by combination with minimal dose pantoprazole	Induction of SHP-1 and inhibition of JAK2/STAT3

PPI: Proton pump inhibitor; V-H^+-ATPase: Vacuolar-H^+-ATPase; LRP6: Low-density lipoprotein receptor related protein 6; MDR: Multidrug resistance; MRP1: Multidrug resistance-associated protein 1; mTOR: Mammalian target of rapamycin; HIF-1α: Hypoxia-inducible factor 1alpha; CAF: Cancer associated fibroblast; FOXO1: Forkhead box protein O1; TGF-beta: Tumor growth factor-beta; EMT: Epithelial-mesenchymal transition; STAT3: Signal transducer and activator of transcription 3; JAK2: Janus kinase 2; ADR: Adriamycin; GSK-β: Glycogen synthetase kinase-3-β.

CONCLUSION

Many physicians have raised concerns that long-term PPI use may be a significant risk factor for GI tract neoplasia, including gastric cancer, and data from recent clinical studies support this hypothesis. However, from a methodological point of view, application of the results from observational clinical studies is limited until solid evidence is available to establish the long-term use of PPI and its association with gastric cancer. However, in patients with pre-malignant lesions such as atrophic gastritis or intestinal metaplasia, it may be necessary to restrict long-term PPI administration, even after *H. pylori* eradication, to prevent gastric cancer. By contrast, theoretical investigations and experimental findings suggest that PPIs may play an

adjunct role of in improving the efficacy of chemotherapy for malignant tumors including stomach cancer. Currently, PPIs might play a "dual role" in gastric carcinogenesis and management of advanced gastric cancer.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 May 7; 25(17): 2071-2085

DOI: 10.3748/wjg.v25.i17.2071

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Herbs-partitioned moxibustion alleviates aberrant intestinal epithelial cell apoptosis by upregulating A20 expression in a mouse model of Crohn's disease

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Author contributions: Zhou J, Shi Y, and Wu HG designed the research; Zhou J, Chen L, Zhao JM, Guo YJ, Sun Y, and Li T performed the experiments; Wu LY, Zhou J, and Bao CH collected and analyzed the data; Zhou J wrote the manuscript; all authors reviewed the manuscript prior to its submission, and read and approved the final manuscript.

Supported by National Natural Science Foundation of China, No. 81273844 and No. 81473757; National Key Basic Research Program of China (973 Program), No. 2015CB554500; and Shanghai Rising-Star Program, No. 16QA1403400.

Institutional animal care and use committee statement: All animal experiments in this study were performed under guidelines approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine (No. 2013025).

Conflict-of-interest statement: The

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Abstract

BACKGROUND

A20 inhibits intestinal epithelial cell apoptosis in Crohn's disease, and herbs-partitioned moxibustion (HPM) has been demonstrated to be an effective treatment for Crohn's disease. However, the mechanism by which HPM reduces intestinal epithelial cell apoptosis in Crohn's disease has not been thoroughly elucidated to date.

AIM

To elucidate whether HPM exerts its effects by upregulating A20 to affect intestinal epithelial cell apoptosis in a Crohn's disease mouse model.

METHODS

In this study, mice with A20 deletion in intestinal epithelial cells ($A20^{IEC\text{-}KO}$) were utilized to establish a Crohn's disease mouse model with 2,4,6-trinitrobenzene

authors declare no conflicts of interest.

Data sharing statement: No additional data are available.

ARRIVE guidelines statement: The manuscript was prepared according to the ARRIVE guidelines.

Manuscript source: Unsolicited manuscript

Received: January 13, 2019
Peer-review started: January 14, 2019
First decision: February 13, 2019
Revised: March 13, 2019
Accepted: March 15, 2019
Article in press: March 16, 2019
Published online: May 7, 2019

P-Reviewer: Abdolghaffari AH
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

sulfonic acid (TNBS) administration, as well as wild-type mice. Mice were randomly divided into normal control (NC), model control (MC), mesalazine (MESA), and HPM groups. The morphology of the colonic mucosa was observed by hematoxylin-eosin staining, and serum endotoxin and apoptosis of epithelial cells were evaluated by enzyme-linked immunosorbent assay and terminal dUTP nick-end labeling assay accordingly. The protein expression levels of A20 and tumor necrosis factor receptor 1 (TNFR1)-related signaling molecules were evaluated by Western blot, and co-expression of A20 and TNFR1-associated death domain (TRADD) and co-expression of A20 and receptor-interacting protein 1 (RIP1) were observed by double immunofluorescence staining.

RESULTS

The intestinal epithelial barrier was noted to have an improvement in the HPM group of wild-type (WT) mice compared with that in $A20^{IEC\text{-}KO}$ mice. Compared with $A20^{IEC\text{-}KO}$ HPM mice, serum endotoxin levels and apoptosis percentages were decreased ($P < 0.01$), A20 expression levels were increased ($P < 0.01$), and expression of TNFR1, TRADDD, and RIP1 was decreased in the HPM group of WT mice ($P_{TNFR1} < 0.05$, $P_{TRADD} < 0.01$, $P_{RIP1} < 0.01$). Both of the co-expression of A20/TRADD and A20/RIP1 showed a predominantly yellow fluorescence in the HPM group of WT mice, while a predominantly red fluorescence was noted in the HPM group of $A20^{IEC\text{-}KO}$ mice.

CONCLUSION

Our findings suggest that HPM in treating Crohn's disease functions possibly via upregulation of the A20 expression level, resulting in downregulation of TNFR1, TRADD, and RIP1 to alleviate increased cell apoptosis in the intestinal epithelial barrier in Crohn's disease.

Key words: Herbs-partitioned moxibustion; Crohn's disease; Apoptotic pathway; Inflammation; A20

Core tip: We report our results derived from mice with A20 deletion in intestinal epithelial cells by inducing Crohn's disease. The Crohn's disease model was induced with 2,4,6-trinitrobenzene sulfonic acid. This study demonstrates for the first time that herbs-partitioned moxibustion can upregulate the expression of A20, resulting in downregulation of tumor necrosis factor receptor (TNFR) 1, TNFR1-associated death domain, and receptor-interacting protein 1 to alleviate increased cell apoptosis in the intestinal epithelial barrier in Crohn's disease.

Citation: Zhou J, Wu LY, Chen L, Guo YJ, Sun Y, Li T, Zhao JM, Bao CH, Wu HG, Shi Y. Herbs-partitioned moxibustion alleviates aberrant intestinal epithelial cell apoptosis by upregulating A20 expression in a mouse model of Crohn's disease. *World J Gastroenterol* 2019; 25(17): 2071-2085
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2071.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2071

INTRODUCTION

Crohn's disease is a chronic inflammatory bowel disease with symptoms of abdominal pain, diarrhea, weight loss, perianal lesions, and anemia[1]. In the past ten years, Crohn's disease has increased significantly as a worldwide health problem[2]. The highest reported prevalence areas are Europe and North America with 322 cases per 100000[3,4]. The high prevalence and long-term nature of the disease pose a great burden on patients and the society, due to health-related reduction in quality of life and decreased economic productivity, which calls for high-quality and cost-efficient care for patients.

Crohn's disease most likely results from complex interactions between genetics, environment, and gut microbiota, which lead to dysfunction of the epithelial barrier

with consequent deregulation of the mucosal immune system and responses to gut microbiota[5,6]. The intestinal epithelium is a notably large mucosal surface with a strong capacity for self-renewal[7,8]. Proliferation of progenitor cells and their differentiation into mature epithelial cells continuously recompense cell losses[9]. However, in Crohn's disease, this homeostasis is disrupted by increased apoptosis of epithelial cells due to increased stimulation of the immune system, leading to loss of epithelial integrity and local inflammation[10]. In many patients with Crohn's disease, epithelial injury and inflammation due to increased cell apoptosis depend on tumor necrosis factor (TNF)[11]. TNF-alpha (TNF-α), a transmembrane protein, binds to its ligand, TNF receptor (TNFR) 1, recruiting TNFR1-associated death domain (TRADD) and receptor-interacting protein (RIP). In turn, TRADD and RIP associate with FAS-associated death domain protein (FADD) to activate caspase 8, leading to apoptosis[12]. Studies have shown that lower expression of A20 correlates with improved anti-TNF-α drug responses[13], and mice with A20 deletion in intestinal epithelial cells ($A20^{IEC\text{-}KO}$) are hypersensitive to TNF-α-induced intestinal epithelial apoptosis[14]. These studies have revealed a negative regulatory role of A20 in the TNF-α-induced apoptosis pathway.

A20 is a cytoplasmic protein that was originally identified as a primary TNF-α-induced responsive molecule in endothelial cells and negatively regulates NF-κB-dependent gene expression in response to stimuli, such as TNF-α and interleukin-1 (IL-1)[15,16]. A20 contains an N-terminal ovarian tumor deubiquitinating and E3 ubiquitin ligase activity toward the death-domain-containing protein kinase RIP1 and adaptor proteins in the TNF-α/TNFR1 pathway, and is the most important anti-apoptotic protein involved in diseases such as Crohn's disease[17,18] and glioblastoma[19]. A20 inhibits TNF-α-induced apoptosis by disrupting recruitment of TRADD and RIP1 to the TNFR1 complex[19]. It has been reported that $A20^{IEC\text{-}KO}$ mice are normal, but they are more likely to suffer from intestinal injury induced by intraperitoneal TNF-α injection[13,14]. Furthermore, clinical studies show that the mucosal expression of A20 was significantly lower in Crohn's disease patients compared to healthy controls[13,17]. Taken together, these findings indicate that A20 expression levels are critical in maintaining epithelial barrier function, which may provide a molecular mechanism for illustrating apoptosis in the development of Crohn's disease.

Despite the effectiveness of Western medications, such as aminosalicylates and thiopurines, in inducing and maintaining remission in Crohn's disease, they are limited by their serious side effects, such as nausea, bone marrow suppression, hepatitis, allergic reaction, pancreatitis, and infections and opportunistic infections[20-24]. Approximately 10%-26% of patients withdraw from these treatments because of the adverse effects[23,25]. Thus, a safer therapy is necessary for managing the disease. Moxibustion, a traditional Chinese medicine, has been demonstrated to be an effective and safe method in treating mild and moderate active-phase Crohn's disease with long-term clinical efficacy[26-28]. After 12 wk of herbs-partitioned moxibustion (HPM) therapy, 74% of 46 mild and moderate Crohn's disease patients [Crohn's disease Activity Index (CDAI) from 151 to 350] entered remission periods (CDAI scores < 150). In addition, moxibustion can effectively relieve symptoms such as abdominal pain and diarrhea and can increase hemoglobin counts and decrease C-reactive protein levels[28]. No adverse events were reported in these studies. We previously demonstrated that HPM improved intestinal epithelial barrier function by downregulating the apoptosis of intestinal epithelial cells[29].

However, whether HPM regulates A20 expression to downregulate intestinal epithelial cell apoptosis in Crohn's disease is obscure. Thus, we used HPM and the most frequently prescribed medication, mesalazine (an aminosalicylate)[24], in both $A20^{IEC\text{-}KO}$ and wild-type (WT) Crohn's disease mice to evaluate the efficacy of HPM in upregulating A20 expression in an apoptotic pathway induced by TNF-α/TNFR1.

MATERIALS AND METHODS

Animals

Eight-week-old $A20^{IEC\text{-}KO}$ and WT C57BL/6 mice were obtained from the Shanghai Model Organisms Center (Shanghai, China). The mice were housed at the animal care center of the Shanghai University of Traditional Chinese Medicine and were provided with humane care in a temperature-controlled room (temperature of 22 ± 1 °C and humidity 50% ± 70%) under a 12-h light–dark cycle with free access to food and water. All animal experiments in this study were performed under guidelines approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine (No. 2013025).

Establishment of a mouse model of Crohn's disease

Forty-eight C57BL/6 WT and A20^{IEC-KO} mice each were randomly divided into normal control (NC, n = 12), model control (MC, n = 12), mesalazine (MESA, n = 12), and HPM (n = 12) groups. The MC, MESA, and HPM groups were administered with 2,4,6-trinitrobenzene sulfonic acid (TNBS) enemas to establish an experimental Crohn's disease model[30]. The enema solution was prepared with absolute ethanol and 5% TNBS at a 1:1 proportion. The solution was stored away from light. Mice were provided access to water only for 24 h prior to TNBS administration and were weighed. Mice were then anesthetized with 0.05 mL/10 g of 1% pentobarbital sodium via intraperitoneal injection. All mice apart from NC group mice were administered TNBS/ethanol (0.06 mL/10 g of TNBS + 50% ethanol 0.25 mL) intra-anally via a rubber tube, and the solution was retained in the gut cavity at a depth of 3-4 cm. NC mice were administered with physiological saline at 0.05 mL/10 g. All mice were fixed in a handstand posture for 2 min after the rubber tube was withdrawn to prevent outflow of solution. This procedure was performed once. Two mice were randomly selected from each group and sacrificed to confirm the presence of Crohn's disease-like intestinal pathology by hematoxylin and eosin stain (H&E) staining after 4 d.

Treatment methods

Upon confirmation of the model establishment, HPM group mice were treated with HPM. Moxa cones (0.5 cm in diameter and 0.3 cm high) made of refined mugwort floss were placed on herbal cakes [*e.g.*, medicinal formula dispensing (radix) Aconiti praeparata, (cortex) Cinnamomi] at Tianshu (ST25) and Qihai (CV6) acupuncture points (which regulate intestinal functions) and ignited. Two moxa cones were used per treatment, which was administered once daily for 10 d. MESA group mice were fed MESA, which was prepared at a proportion of 1:0.0026[31], twice daily for 10 d. Mice in the MC and NC groups did not undergo any treatment.

Histological observation

All mice were sacrificed simultaneously at the conclusion of treatment. Approximately 4 cm of colon lesions were resected at a 3-4 cm distance from the anus. A 1 cm length of the dissected colon was removed, washed with iced saline, fixed in 10% natural buffered formalin solution, embedded in paraffin, cut into tissue sections, and stained with H&E. Obtained images were observed under a light microscope (Olympus, Tokyo, Japan).

Enzyme-linked immunosorbent assay

A 96-well commercial kit (MyBioSource, Inc. San Diego, CA, United States) was applied to evaluate serum endotoxin levels. Blood samples were centrifuged at 3000 × g for 10 min. Diluted serum sample (1:10), endotoxin test water, and TAL were added to the plate and incubated for 10 min at 37 °C. Then, chromogenic matrix solution was added to the plate and incubated for 6 min at 37 °C. After that, azo reagent was added to the plate and incubated for 5 min at 37 °C. Optical densities were detected with a plate reader (BioTek Instruments, Winooski, VT, United States)

Western blot analysis

Protein (60 μg) extracted from isolated rat intestinal epithelial cell samples was separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% skimmed milk in TBS-T for 1 h at room temperature and incubated with the following primary antibodies overnight at 4 °C: A20 (1:1000; ab13597, Abcam), TNF-α (1:1000; ab6671, Abcam), TNFR1 (1:5000; ab19139, Abcam), TRADD (1:500; ab110644, Abcam), RIP1 (1:500; ab72139, Abcam), FADD (1:400; ab24533, Abcam), and GAPDH (1:1500; 5174, CST). Following several sequential washes, the membranes were incubated with the corresponding secondary anti-mouse antibody (A0208, Beyotime) for 1 h at room temperature. Blots were then washed four times with TBS-T (10 min each time). The membranes were stained with ECL enhanced chemiluminescence solution and visualized using a visualizer.

Terminal dUTP nick-end labeling assay

A terminal dUTP nick-end labeling (TUNEL) kit purchased from MyBioSource was utilized to evaluate apoptosis. Paraffin sections were first fully deparaffinized and hydrated, treated with protease K solution (20 μg/mL) for 15 min at room temperature, and subsequently washed and immersed in 3% hydrogen peroxide for 15 min. The sections were then immersed in an equilibrium buffer for 20 min at room temperature and afterward incubated with TdTase for 60 min at 37 °C. After that, the sections were stained with 3,3'-diaminobenzidine (DAB, Shanghai Long Island Biotec.

Co., Ltd, China) and counterstained with hematoxylin. Apoptotic cells as well as the total number of cells were calculated.

Double immunofluorescence staining

Paraffin sections were fully deparaffinized and hydrated, and then washed and heated to 92-98 °C for antigen retrieval. Samples were incubated with primary antibodies against A20 (ab13597, Abcam), RIP1 (ab72139, Abcam), and TRADD (ab110644, Abcam) in blocking buffer overnight at 4 °C. Samples were subsequently incubated with the corresponding secondary antibody in blocking buffer at room temperature, and finally incubated with DAPI staining solution for 10 min. Images were obtained under a fluorescence microscope (Nikon, Japan).

Statistical analysis

Experimental data were analyzed with SPSS 21.0 software (SPSS Inc., Wacker Drive, Chicago, IL, Uinted States) and GraphPad Prism 5 (GraphPad Software, San Diego, CA, Uinted States). All data are presented as the mean ± standard deviation (SD). Statistics among each experimental group were analyzed using one-way analysis of variance (ANOVA) followed by the least significant difference test. The level of significance was set at $a = 0.05$ and $^{a}P < 0.05$, $^{b}P < 0.01$; $^{c}P < 0.05$, $^{d}P < 0.01$; $^{e}P < 0.05$, $^{f}P < 0.01$; $^{g}P < 0.05$, $^{h}P < 0.01$.

RESULTS

Intestinal morphological observations in each group

Previous studies have shown that the Crohn's disease model of A20$^{IEC\text{-}KO}$ mice showed a more severely damaged mucosa than WT mice[13]. In this study, by histopathological evaluation, we found that in WT NC mice, intact mucosal epithelial cells and normal morphological changes affecting the submucosa and muscularis were observed (Figure 1A). In WT MC mice, sparse goblet cells, fibrous hyperplasia, as well as damage to mucosal glands, vasodilation, and hyperemia were noted in the mucosa, and hyperemia and edema were observed under the submucosa (Figure 1B). In WT MESA mice, sparse goblet cells, infiltration of inflammatory cells into the mucosa and submucosa, as well as hyperemia and edema under the submucosa were noted. No obvious abnormal changes were observed in the structural morphology of mucosal, submucosal, and muscularis layers (Figure 1C). In WT HPM mice, sparse goblet cells, mild infiltration of inflammatory cells into the colonic mucosa and submucosa, sparse fibroblast hyperplasia, and healing ulcers were observed. No obvious abnormal changes were observed in the structural morphology of the mucosal, submucosal, or muscularis layer (Figure 1D).

In A20$^{IEC\text{-}KO}$ NC mice, intact mucosal epithelial cells and normal submucosal and muscularis structural morphology were observed (Figure 1E). In A20$^{IEC\text{-}KO}$ MC mice, partial epithelial cells loss, mucosal goblet cell depletion, inflammatory cell infiltration, glandular damage, and proliferation of fibrous tissue were observed in the mucosa in addition to erosion and necrosis of the mucosal surface. Hyperemia and edema were observed in both mucosal and submucosal layers (Figure 1F). In A20$^{IEC\text{-}KO}$ MESA mice, sparse goblet cells, small healing ulcers with associated glandular destruction, and massive inflammatory cell infiltration were observed in the mucosa. No obviously abnormal structural changes in the mucosal, submucosal, or muscularis layer were observed (Figure 1G). In A20$^{IEC\text{-}KO}$ HPM mice, sparse goblet cells, inflammatory cell infiltration, and proliferation of fibrous tissue were observed in mucosal tissue. No obviously abnormal structural changes in the mucosal, submucosal, or muscularis layer were noted (Figure 1H).

Variations in epithelial permeability across groups

Next, we observed the intestinal epithelial barrier permeability by detecting serum endotoxin levels(Figure 2). In WT groups, as compared with NC mice, serum endotoxin levels were upregulated in the MC, MESA, and HPM groups ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Serum endotoxin levels were downregulated in HPM ($P < 0.01$) and MESA ($P < 0.05$) mice as compared to MC mice. Serum endotoxin levels were significantly decreased in HPM mice ($P < 0.01$) as compared to MESA mice.

In mice from A20$^{IEC\text{-}KO}$ groups, as compared with NC mice, serum endotoxin levels were upregulated in the MC, MESA, and HPM groups ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). They were downregulated in MESA and HPM mice as compared to MC mice ($P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Serum endotoxin levels were significantly decreased in HPM mice ($P < 0.01$) as compared to those of the MESA group.

Compared with WT NC mice, no significant difference in serum endotoxin levels were noted in NC A20$^{IEC\text{-}KO}$ mice ($P > 0.05$). Compared with WT MC mice, endotoxin

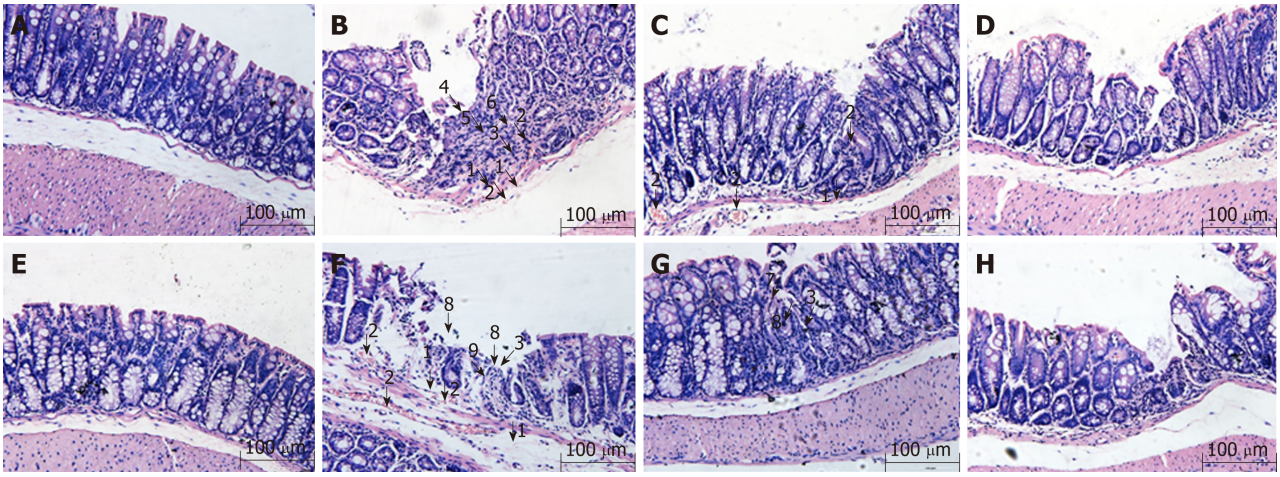

Figure 1 Histological observation of intestinal epithelial tissues across groups (magnification,×100). A: Wild-type mice in normal control group; B: Wild-type mice in model control group; C: Wild-type mice in mesalazine group; D: Wild-type mice in herbs-partitioned moxibustion group; E: A20^{IEC-KO} mice in normal control group; F: A20^{IEC-KO} mice in model control group; G: A20^{IEC-KO} mice in mesalazine group; H: A20^{IEC-KO} mice in herbs-partitioned moxibustion group. 1: Tissue edema; 2: Hyperemia; 3: Inflammatory cell infiltration; 4: Necrosis; 5: Granulation tissue proliferation; 6: Destruction of glandular structure; 7: Healing ulcer; 8: Ulcer; 9: Proliferation of fibrous tissue.

levels in MC A20^{IEC-KO} mice were upregulated ($P < 0.05$). Compared with WT HPM mice, serum endotoxin levels were upregulated in HPM A20^{IEC-KO} mice ($P < 0.01$).

Observation of percentage of apoptotic intestinal epithelial cells in each group

Since A20 protein is involved in epithelial barrier function by its anti-apoptotic role in Crohn's disease, we observed cell apoptosis percentage in different groups by TUNEL method. In WT groups, as compared with NC mice, apoptosis percentages were significantly increased in the MC, MESA, and HPM groups ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HMP} < 0.01$). Apoptosis percentages were significantly decreased in MESA and HPM mice ($PMESA < 0.01$, $PHPM < 0.01$) as compared to those of the MC group. No significant difference in apoptosis percentages was noted in HPM mice ($P > 0.05$) (Figure 3A-D, I) as compared to those of the MESA group.

In A20^{IEC-KO} groups, as compared with NC mice, apoptosis percentages were significantly increased in the MC, MESA, and HPM groups ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Apoptosis percentages were significantly decreased in the MESA and HPM groups ($P_{MESA} < 0.01$, $P_{HPM} < 0.01$) as compared to the MC group. There was no difference between the HPM and MESA groups ($P > 0.05$) (Figure 3E-I).

Compared with each corresponding group of WT mice, apoptosis percentages were significantly increased in the NC, MC, MESA, and HPM groups of A20^{IEC-KO} mice ($P_{NC} < 0.01$, $P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$) (Figure 3A-I).

Expression of members of the TNF-α/TNFR1-TRADD-FADD apoptotic pathway in the intestinal epithelium across groups

A20 expression across groups: In WT groups, as compared with the NC group, A20 levels were decreased in MC, MESA ($P_{MC} < 0.01$, $P_{MESA} < 0.01$), and HPM mice ($P_{HPM} < 0.05$). Compared with MC mice, A20 expression was significantly increased in HPM mice ($P < 0.01$) and increased in MESA mice ($P < 0.05$). Compared with MESA mice, no difference in A20 levels was noted in HPM mice ($P > 0.05$) (Figure 4A). No significant differences in A20 expression was noted among A20^{IEC-KO} groups ($PNC > 0.05$, $PMC > 0.05$, $PMESA > 0.05$, $PHPM > 0.05$) (Figure 4A). Compared with each corresponding group of WT mice, A20 expression levels were significantly decreased in all A20^{IEC-KO} groups ($P_{NC} < 0.01$, $P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$) (Figure 4A).

TNF-α expression across groups: In WT groups, as compared with NC mice, TNF-α levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Compared with MC mice, they were significantly decreased in MESA and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$). No differences in TNF-α expression in HPM mice were found as compared to those of the MESA group ($P > 0.05$) (Figure 4B).

In A20^{IEC-KO} groups, TNF-α levels were significantly increased in the MC, MESA, and HPM groups as compared to those in NC mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HMP} < 0.01$). TNF-α levels were significantly decreased in MESA and HPM mice ($P_{MESA} < 0.01$, $P_{HMP} < 0.01$) as compared to MC mice. TNF-α levels were decreased in HPM mice as

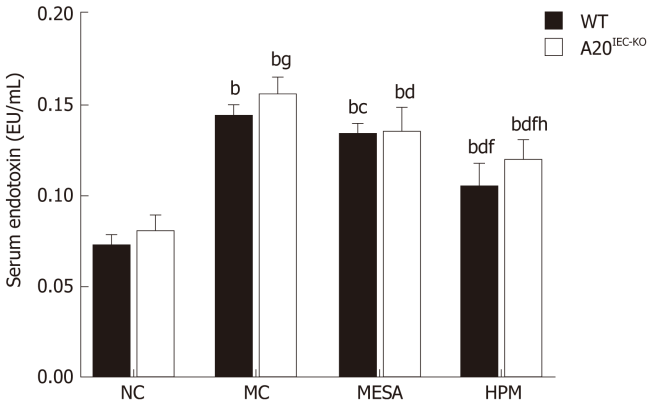

Figure 2 Serum endotoxin levels in mice across groups. Data are presented as the mean ± standard deviation (n = 10). Data were evaluated for statistical significance by one-way analysis of variance and are represented as follows: ^{a}P<0.05, ^{b}P < 0.01 as compared to normal control; ^{c}P < 0.05, ^{d}P < 0.01 as compared to model control; ^{e}P < 0.05, ^{f}P < 0.01 as compared to mesalazine; ^{g}P < 0.05, ^{h}P < 0.01 as compared to wild type. WT: Wild type; NC: Normal control; MC: Model control; MESA: Mesalazine; HPM: Herbs-partitioned moxibustion.

compared to those of the MESA group (P < 0.05) (Figure 4B). Compared with each corresponding group of WT mice, TNF-α levels in the A20^{IEC-KO} NC and HPM groups were not different (P > 0.05); TNF-α expression was increased in MC mice (P < 0.05) and significantly increased in MESA mice (P < 0.01) (Figure 4B).

TNFR1 expression across groups: In WT groups, as compared with NC mice, TNFR1 expression was significantly increased in MC, MESA, and HPM mice (P_{MC} < 0.01, P_{MESA} < 0.01, P_{HPM} < 0.05). Compared with MC mice, TNFR1 expression was significantly decreased in mice of the MESA and HPM groups (P_{MESA} < 0.01, P_{HMP} < 0.01). No difference in TNFR1 expression was found in HPM mice as compared to those of the MESA group (P > 0.05) (Figure 4C). In the A20^{IEC-KO} groups, as compared with NC mice, TNFR1 expression was significantly increased in MC and HPM mice (P_{MC} < 0.01, P_{HPM} < 0.01). It was increased in MESA mice as well (P_{MESA} < 0.05). Compared with MC mice, TNFR1 expression was significantly decreased in mice of the MESA and HPM groups (P_{MESA} < 0.01, P_{HMP} < 0.01). No difference in TNFR1 expression in mice of the HPM group was noted as compared to that in the MESA group (P > 0.05) (Figure 4C). Compared with the corresponding WT MC and WT HPM groups, TNFR1 expression was increased in A20^{IEC-KO} mice (PMC < 0.05, $PHPM$ < 0.05). No difference in TNFR1 expression among corresponding WT and A20^{IEC-KO} NC and MESA groups was noted (PNC > 0.05, $PMESA$ > 0.05) (Figure 4C).

TRADD expression across groups: In WT groups, as compared with NC mice, TRADD expression was significantly increased in MC and MESA mice (P_{MC} < 0.01, P_{MESA} < 0.01) and increased in HPM mice (P_{HMP} < 0.05). TRADD levels were significantly decreased in mice of the HPM group (P < 0.01) and decreased in those of the MESA group (P < 0.05) as compared to those of the MC group. Compared with MESA group mice, no difference in TRADD expression in HPM group mice was noted (P > 0.05) (Figure 4D). In A20^{IEC-KO} groups, compared with NC mice, TRADD levels were significantly increased in MC, MESA, and HPM mice (P_{MC} < 0.01, P_{MESA} < 0.01, P_{HPM} < 0.01). No difference in TRADD levels among the MC, MESA, and HPM groups was noted (P > 0.05) (Figure 4D). Compared with WT NC mice, no difference in TRADD expression was found in the same group of A20^{IEC-KO} mice (P > 0.05). TRADD expression was found to be significantly increased in MESA and HPM group mice (P_{MESA} < 0.01, P_{HPM} < 0.01) and increased in MC group A20^{IEC-KO} mice (P < 0.05) when compared to corresponding WT groups (Figure 4D).

RIP1 expression across groups: In WT groups, compared with NC group mice, RIP1 levels were significantly increased in the MC, MESA, and HPM groups (P_{MC} < 0.01, P_{MESA} < 0.01, P_{HPM} < 0.01). Compared with MC group mice, RIP1 levels were decreased in those of the HPM group (P < 0.05); no difference in RIP1 levels (P > 0.05) was noted in MESA group mice. Compared with MESA group mice, no difference in RIP1 levels was noted in those of the HPM group (P > 0.05) (Figure 4E). In mice of A20^{IEC-KO} groups, compared with those of the NC group, RIP1 levels were significantly increased in MC, MESA, and HPM mice (P_{MC} < 0.01, P_{MESA} < 0.01, P_{HPM} < 0.01). No differences in RIP1 levels among the MC, MESA, and HPM groups were noted (P > 0.05) (Figure 4E). Compared with WT NC mice, no difference in RIP1 expression in

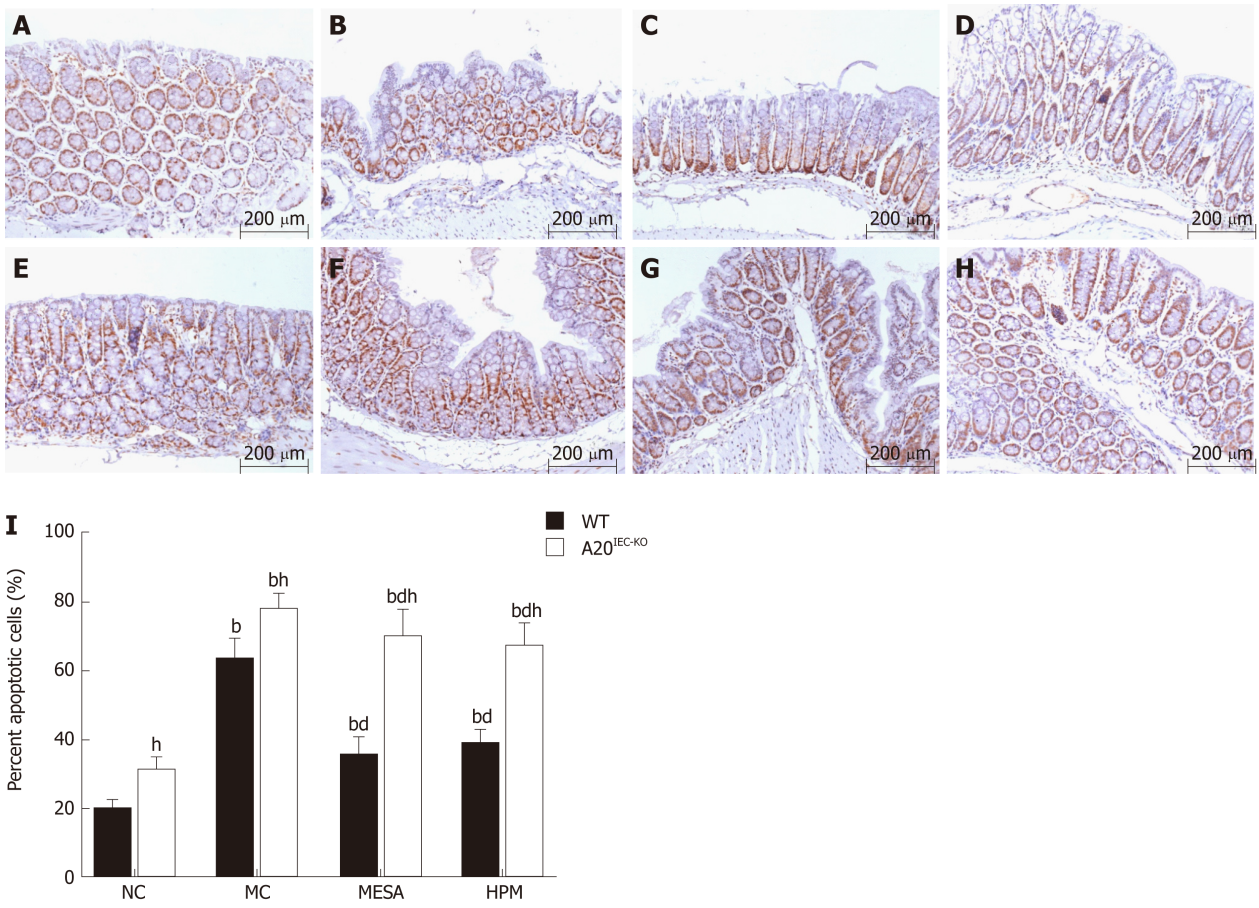

Figure 3 Apoptosis percentages of intestinal epithelial cells across groups (magnification,×200). A: Wild-type mice in normal control group; B: Wild-type mice in model control group; C: Wild-type mice in mesalazine group; D: Wild-type mice in herbs-partitioned moxibustion; E: A20^{IEC-KO} mice in normal control group; F: A20^{IEC-KO} mice in model control group; G: A20^{IEC-KO} mice in mesalazine group; H: A20^{IEC-KO} mice in herbs-partitioned moxibustion group; I: Percentage of apoptotic cells. Data are presented as the mean ± standard deviation (n = 10). Data were evaluated for statistical significance by one-way analysis of variance and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to normal control; $^{c}P < 0.05$, $^{d}P < 0.01$ as compared to model control; $^{e}P < 0.05$, $^{f}P < 0.01$ as compared to mesalazine; $^{g}P < 0.05$, $^{h}P < 0.01$ as compared to wild type. WT: Wild type; NC: Normal control; MC: Model control; MESA: Mesalazine; HPM: Herbs-partitioned moxibustion.

A20^{IEC-KO} NC mice ($P > 0.05$) was found. Compared with WT HPM mice, expression of RIP1 was increased in A20^{IEC-KO} HPM mice ($P < 0.01$). Compared with corresponding WT MC and MESA mice, RIP1 expression was increased in A20^{IEC-KO} mice ($P_{MC} < 0.05$, $P_{MESA} < 0.05$) (Figure 4E).

FADD expression across groups: In WT groups, compared with NC mice, FADD levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Compared with MC mice, FADD expression was decreased in MESA and HPM mice ($P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Compared with mice of the MESA group, no difference in FADD expression levels was found in HPM mice ($P > 0.05$) (Figure 4F). In the A20^{IEC-KO} groups, compared with NC mice, FADD levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). No difference in FADD expression was noted among MC, MESA, and HPM mice ($P > 0.05$) (Figure 4F). Compared with WT NC and MC mice, no difference in FADD levels was found between A20^{IEC-KO} NC and MC mice ($PNC > 0.05$, $PMC > 0.05$). Compared with WT HPM mice, FADD levels were significantly increased in A20^{IEC-KO} HPM mice ($P < 0.01$). Compared with WT MESA mice, FADD levels were increased in A20^{IEC-KO} MESA mice ($P < 0.05$) (Figure 4F).

Co-expression of A20/TRADD and A20/RIP1 in intestinal epithelial tissues across groups

Co-expression of A20/TRADD: Cell nuclei stained blue. Regions rich in A20 expression stained green while those rich in TRADD stained red. Co-expression of A20 and TRADD stained yellow (red and green fluorescence). In WT NC mice, green fluorescence predominated (Figure 5A). In WT MC mice, red fluorescence predominated along with sparse yellow staining (Figure 5B). In WT MESA mice,

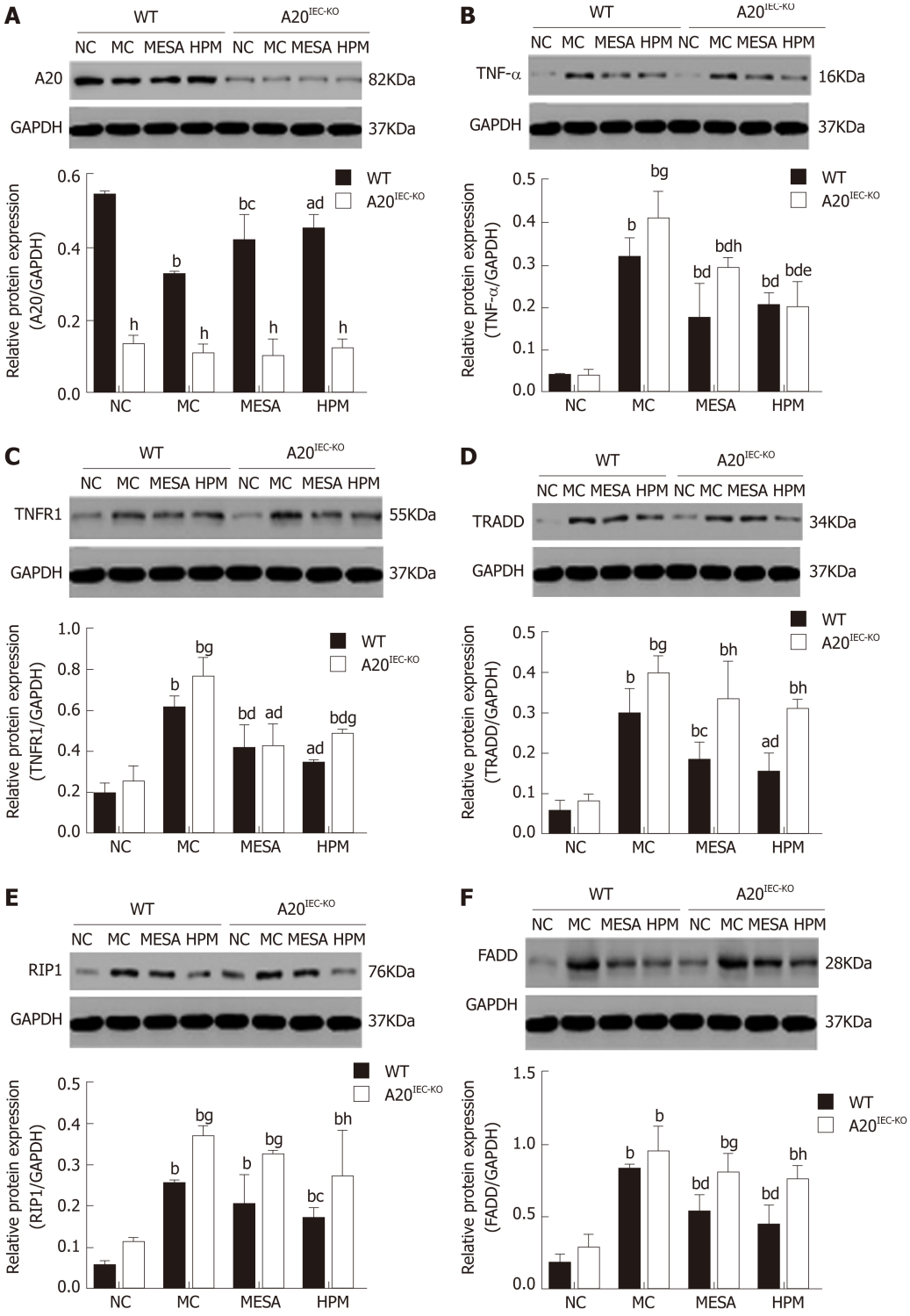

Figure 4 Expression levels of A20, tumor necrosis factor alpha, tumor necrosis factor receptor 1, tumor necrosis factor receptor 1-associated death domain, receptor-interacting protein 1, and FAS-associated death domain protein across groups. Data are presented as the mean ± standard deviation (n = 10). Data were evaluated for statistical significance using one-way analysis of variance and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to normal contro; $^{c}P < 0.05$, $^{d}P < 0.01$ as compared to model control; $^{e}P < 0.05$, $^{f}P < 0.01$ as compared to mesalazine; $^{g}P < 0.05$, $^{h}P < 0.01$ as compared to wild type. TNFR1: Tumor necrosis factor receptor 1; RIP1: Receptor-interacting protein 1; TNF-α: Tumor necrosis factor alpha; TRADD: Tumor necrosis factor receptor 1-associated death domain; FADD: FAS-associated death domain; WT: Wild type; NC: Normal control; MC: Model control; MESA: Mesalazine; HPM: Herbs-partitioned moxibustion.

sparse yellow fluorescence predominated (Figure 5C). In WT HPM mice, immunofluorescence mainly revealed yellow fluorescence (Figure 5D). In A20^{IEC-KO} NC, MC, MESA, and HPM mice, red fluorescence predominated (Figure 5E-H).

In WT groups, compared with NC mice, A20 levels were significantly decreased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). Compared with MC mice, expression of A20 was significantly increased in HPM mice ($P < 0.01$) and

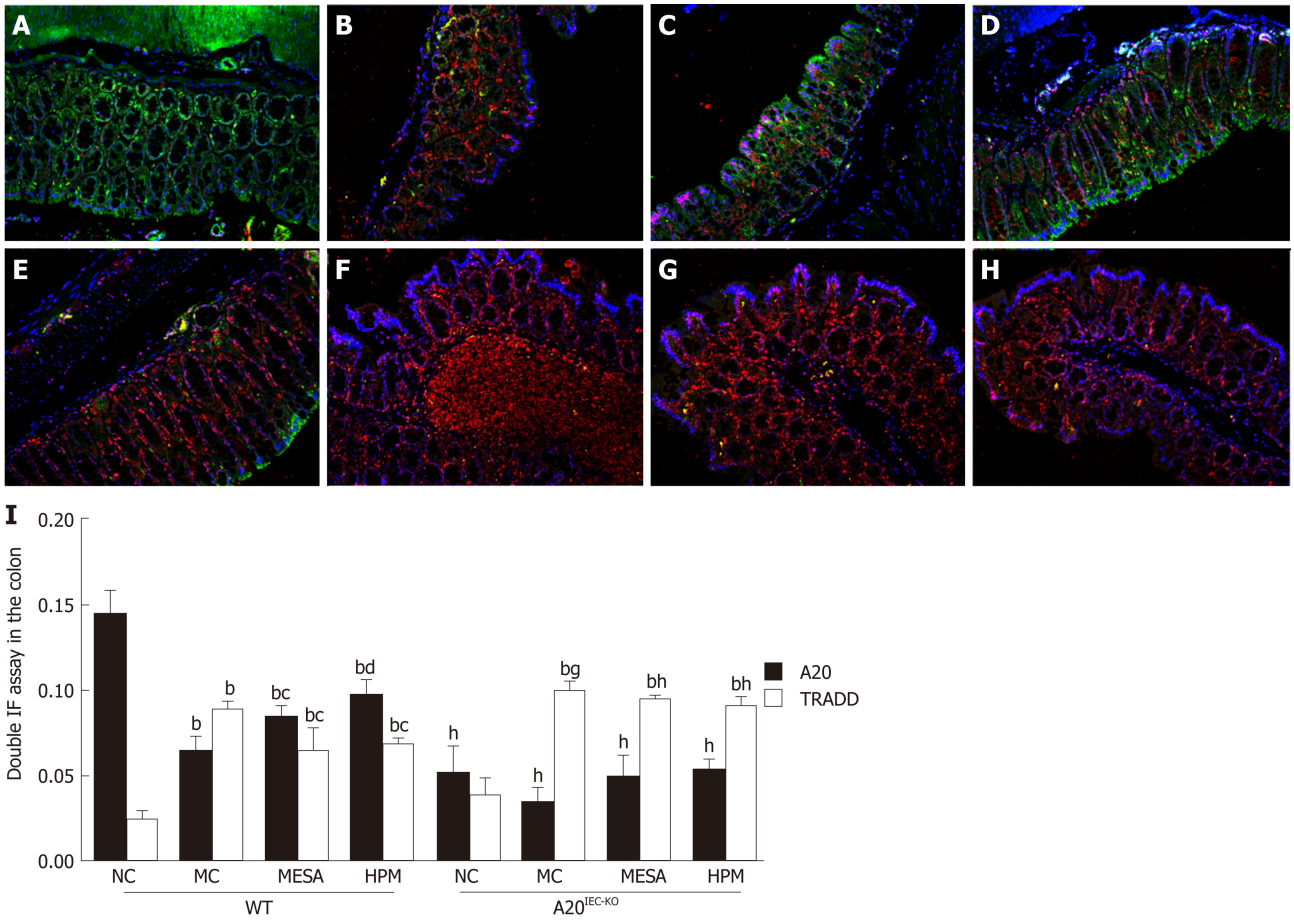

Figure 5 Co-expression of A20/tumor necrosis factor receptor 1-associated death domain in the intestinal epithelium of mice across groups. A: Wild-type mice in normal control group; B: Wild-type mice in model control group; C: Wild-type mice in mesalazine group; D: Wild-type mice in herbs-partitioned moxibustion group; E: A20^{IEC-KO} mice in normal control group; F: A20^{IEC-KO} mice in model control group; G: A20^{IEC-KO} mice in mesalazine group; H: A20^{IEC-KO} mice in herbs-partitioned moxibustion group. Data are presented as the mean ± standard deviation (n = 10). Data were evaluated for statistical significance using one-way analysis of variance and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to normal contro; $^{c}P < 0.05$, $^{d}P < 0.01$ as compared to model control; $^{e}P < 0.05$, $^{f}P < 0.01$ as compared to mesalazine; $^{g}P < 0.05$, $^{h}P < 0.01$ as compared to wild type. TRADD: Tumor necrosis factor receptor 1-associated death domain; WT: Wild type; NC: Normal control; MC: Model control; MESA: Mesalazine; HPM: Herbs-partitioned moxibustion.

increased in those of the MESA group ($P < 0.05$). Compared with MESA mice, no difference in A20 levels in those of HPM mice was noted ($P > 0.05$) (Figure 5I). In A20^{IEC-KO} mice, no difference of A20 levels was noted among each group ($P > 0.05$) (Figure 5I). Compared with each corresponding group of WT mice, expression of A20 was significantly decreased in A20^{IEC-KO} mice ($P < 0.01$) (Figure 5I).

In WT groups, compared with NC mice, TRADD levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HMP} < 0.01$). Compared with MC mice, TRADD levels were decreased in mice of the MESA and HPM groups ($P_{MESA} < 0.05$, $P_{HPM} < 0.05$). Compared with MESA mice, no difference in TRADD levels was found in HPM mice ($P > 0.05$) (Figure 5I). In A20^{IEC-KO} groups, compared with NC mice, TRADD levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). No difference in TRADD levels were noted among MC, MESA, and HPM mice ($P > 0.05$) (Figure 5I). Compared with WT MC mice, TRADD levels were increased in A20^{IEC-KO} MC mice ($P < 0.05$). Compared with WT HPM and MESA mice, TRADD levels were significantly increased in mice of corresponding A20^{IEC-KO} groups ($P_{HPM} < 0.01$, $P_{MESA} < 0.01$) (Figure 5I).

Co-expression of A20/RIP1: Figure 6A-H shows that the nuclei exhibited blue fluorescence while regions rich in A20 and RIP1 stained green and red, respectively. Regions co-expressing A20 and RIP1 mainly stained yellow. In WT NC mice, imaged regions mainly stained green, occasionally intermixed with yellow fluorescence (Figure 6A). In WT MC mice, imaged regions stained mainly red and yellow (Figure 6B). In WT MESA mice, imaged regions mainly stained green (Figure 6C). In WT HPM mice, imaged regions mainly stained yellow (Figure 6D). In A20^{IEC-KO} NC mice, sparse red and green fluorescence was apparent (Figure 6E). In A20^{IEC-KO} MC, MESA, and HPM group mice, red fluorescence predominated (Figure 6F-H).

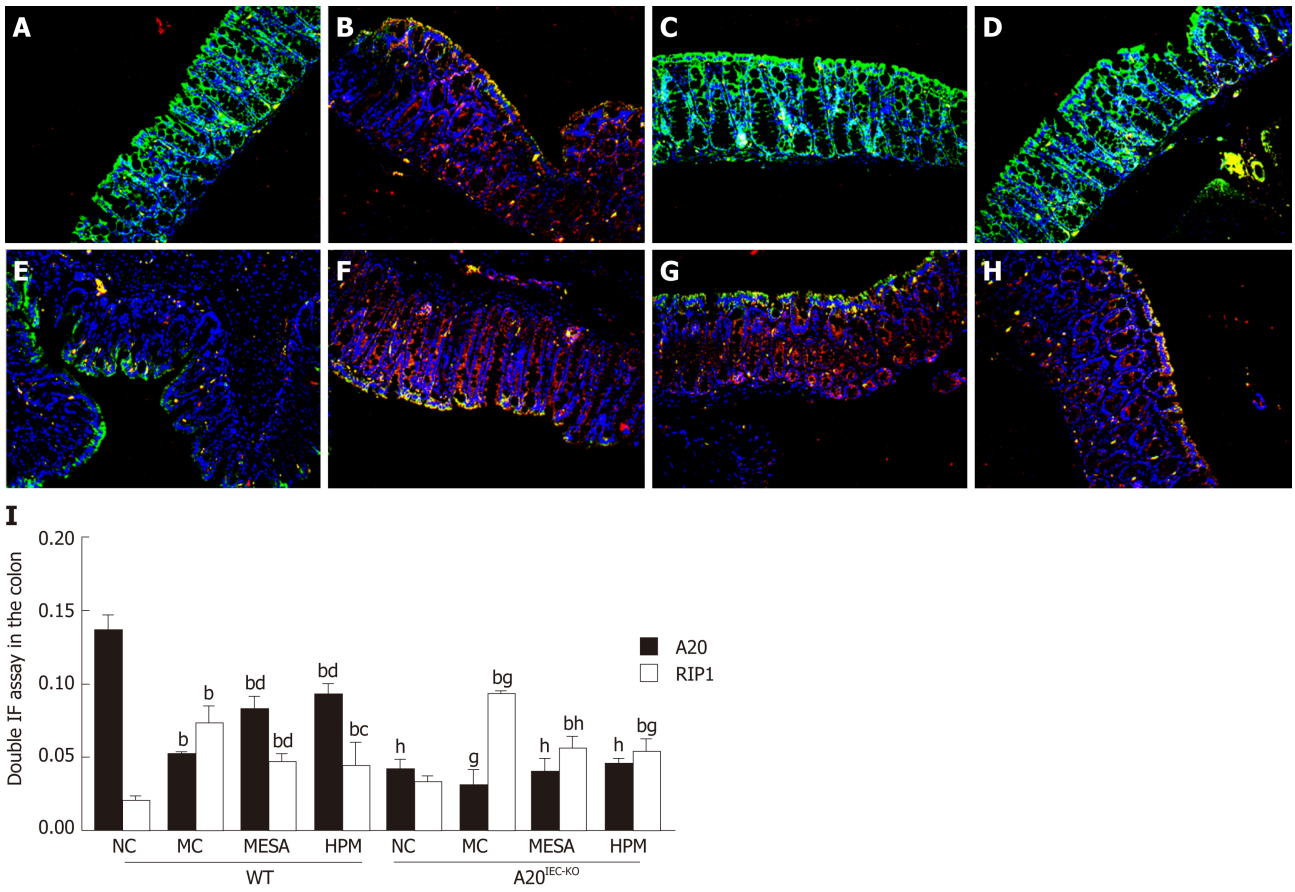

Figure 6 Co-expression of A20/receptor-interacting protein 1 in the intestinal epithelium of mice across groups. A: Wild-type mice in normal control group; B: Wild-type mice in model control group; C: Wild-type mice in mesalazine group; D: Wild-type mice in herbs-partitioned moxibustion group; E: A20^{IEC-KO} mice in normal control group; F: A20^{IEC-KO} mice in model control group; G: A20^{IEC-KO} mice in mesalazine group; H: A20^{IEC-KO} mice in herbs-partitioned moxibustion group. Data are presented as the mean ± standard deviation (n = 10). Data were evaluated for statistical significance using one-way analysis of variance and are represented as follows: $^{a}P < 0.05$, $^{b}P < 0.01$ as compared to normal control; $^{c}P < 0.05$, $^{d}P < 0.01$ as compared to model control; $^{e}P < 0.05$, $^{f}P < 0.01$ as compared to mesalazine; $^{g}P < 0.05$, $^{h}P < 0.01$ as compared to wild type. RIP1: Receptor-interacting protein 1; WT: Wild type; NC: Normal control; MC: Model control; MESA: Mesalazine; HPM: Herbs-partitioned moxibustion.

In WT groups, compared with NC mice, A20 expression was significantly decreased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). A20 levels were significantly increased in HPM and MESA mice ($P_{HPM} < 0.01$, $P_{MESA} < 0.01$) as compared to MC mice. Compared with MESA mice, no difference in levels of A20 were found in HPM mice ($P > 0.05$). In A20^{IEC-KO} groups, no differences in A20 levels were found among groups ($P > 0.05$). Compared with each corresponding group of WT mice, A20 levels were significantly decreased in A20^{IEC-KO} NC, MESA, and HPM mice ($P_{NC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$) and decreased in MC mice ($P_{MC} < 0.05$) (Figure 6I).

In WT groups, compared with NC mice, RIP1 levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HMP} < 0.01$). Compared with MC mice, RIP1 levels were significantly decreased in MESA mice ($P < 0.01$) and decreased in HPM mice ($P < 0.05$). Compared with MESA mice, no difference in RIP1 levels were noted in HPM mice ($P > 0.05$) (Figure 6I). In A20^{IEC-KO} groups, compared with NC mice, RIP1 levels were significantly increased in MC, MESA, and HPM mice ($P_{MC} < 0.01$, $P_{MESA} < 0.01$, $P_{HPM} < 0.01$). No difference in RIP1 levels was found among MC, MESA, and HPM mice ($P > 0.05$) (Figure 6I). Compared with WT NC mice, no difference in RIP1 levels was found in A20^{IEC-KO} NC mice ($P > 0.05$). Compared with WT MC mice, RIP1 expression was increased in A20^{IEC-KO} MC mice ($P < 0.05$). Compared with WT MESA mice, RIP1 expression was significantly increased in mice of the corresponding A20^{IEC-KO} group ($P_{MESA} < 0.01$). Compared with WT HPM mice, RIP1 levels were increased in A20^{IEC-KO} HPM mice ($P_{HPM} < 0.05$) (Figure 6I).

DISCUSSION

Although the etiology of Crohn's disease is still unknown, excessive apoptosis of intestinal epithelial cells leads to villus atrophy and epithelial destruction, which plays a central role in the pathogenesis of the disease[32,33]. A20, as an intestinal epithelium protector, is widely known for its functions in maintaining the epithelial barrier stability in inflammatory conditions. In Crohn's disease patients, there is an excessive inflammatory response and insufficient upregulation of A20 expression[34]. Our previous studies have indicated that HPM plays a beneficial role in Crohn's disease by decreasing intestinal epithelium apoptosis[29]. However, whether the effect of HPM is through A20 has not been determined. In the present study, we examined the anti-apoptotic properties of A20, confirmed its protective role in the intestinal epithelial barrier, and explored whether the effect of HPM in reducing intestinal epithelium apoptosis is through upregulating A20 levels in apoptotic signaling in a TNBS-induced Crohn's disease mouse model in WT and A20^{IEC-KO} lineages.

Aberrant apoptosis of intestinal epithelial cells lacking the A20 gene leads to increased intestinal epithelial permeability in Crohn's disease patients[35-37]. In the present study, we found that the intestinal epithelial cell apoptosis percentage was significantly increased in the Crohn's disease model of A20^{IEC-KO} mice ($P < 0.01$); moreover, serum endotoxin level was upregulated in this lineage ($P < 0.05$). Accordingly, H&E staining analysis showed that the intestinal epithelial barrier was severely damaged in the Crohn's disease model of A20^{IEC-KO} mice, consistent with the Lars Vereecke's report[13]. A previous study showed that HPM reduces intestinal epithelial apoptosis in a Crohn's disease mouse model[29]. The present study indicated that after treatment with HPM, despite improved intestinal morphological changes with decreased intestinal epithelial cell apoptosis percentages and endotoxin levels in A20^{IEC-KO} mice, a more significant improvement was detected in WT mice compared with A20^{IEC-KO} mice ($P < 0.01$). These findings suggested that upregulated A20 can protect intestinal epithelial barrier function and initially confirmed that HPM can upregulate A20 expression to protect the intestinal epithelial barrier in Crohn's disease.

The anti-apoptotic function of A20 has been found to inhibit the sequential signaling complexes of the TNF-α/TNFR1 apoptotic pathway upstream of caspase 8[38]. When TNFR1 binds to TNF-α, the death domain of TNFR1 enables the recruitment of TRADD and RIP1 proteins and their assembly with FADD to activate caspase-8 and induce apoptosis[39-41]. Therefore, we measured the protein expression levels of TNF-α, TNFR1, TRADD, RIP1, and FADD in the apoptotic pathway. The present study found no differences in TNF-α, TNFR1, TRADD, RIP1, or FADD levels among A20^{IEC-KO} NC mice, revealing that A20^{IEC-KO} NC intestinal epithelial cells do not spontaneously undergo apoptosis, in agreement with prior research by Lars Vereecke *et al*[13]. We previously reported that HPM and mild moxibustion downregulate TNF-α and TNFR1 expression levels and decrease intestinal epithelial cell apoptosis in Crohn's disease[29]. Here, we found that when A20 was upregulated in WT mice, TNF-α expression was decreased after HPM treatment in both WT and A20^{IEC-KO} Crohn's disease mice. Interestingly, TNF-α expression levels were not different in A20^{IEC-KO} and WT HPM mice. These results may indicate that HPM could downregulate TNF-α, which is consistent with our previous study[29], but the mechanism enacted by HPM in regulating TNF-α is not specifically associated with A20. TNFR1, TRADD, and RIP1 levels in A20^{IEC-KO} MC mice were found to be increased compared with those in WT mice, suggesting that A20^{IEC-KO} mice are hypersensitive to TNF-α-induced intestinal epithelial apoptosis, consistent with Vereecke's research[14]. Western blot analysis revealed that HPM significantly decreased the expression levels of TNFR1, TRADD, RIP1, and FADD in WT mice but had no effect on the levels of those proteins in A20^{IEC-KO} mice. All of these results indicated that although HPM can downregulate the expression levels of TNFR1, TRADD, RIP1, and FADD, the upregulated expression of A20 by HPM can downregulate TNFR1, TRADD, and RIP1 signaling molecules in the apoptotic pathway.

As TRADD and RIP1 play critical roles in the TNFR1-related signaling apoptotic pathway, we further observed the role of A20 in affecting TRADD and RIP1 expression[12]. A study showed that ligand-dependent association of RIP1 with TNFR1 was significantly reduced in A20-expressing cells in TNF-α-induced apoptosis. Furthermore, the recruitment of TRADD to the TNFR1 complex was also inhibited by A20[42]. In the present study, immunofluorescence showed a predominantly green color with a few yellow areas in intestinal epithelial tissue of the WT HPM group. The imaging data revealed co-expression of A20/TRADD and A20/RIP1 in WT HPM mice and decreased expression of TRADD and RIP1 along with increased expression of A20 in WT HPM mice. In A20^{IEC-KO} HPM mice, immunofluorescence showed a predominantly red color in intestinal epithelial tissue, suggesting a significant amount of TRADD and RIP1 expression without A20 expression. These results may identify a close association of A20, TRADD, and RIP1 within the TNF-α/TNFR1 apoptotic

pathway in a Crohn's disease mouse model. Our findings indicated that HPM upregulates the A20 level, which may affect the expression levels of TRADD and RIP1 in the apoptotic pathway.

In conclusion, HPM in treating Crohn's disease functions possibly via upregulation of the A20 expression level, resulting in downregulation of TNFR1, TRADD, and RIP1 to alleviate increased cell apoptosis in the intestinal epithelial barrier in Crohn's disease.

ARTICLE HIGHLIGHTS

Research background

A20, as an intestinal epithelium protector, plays a critical role in anti-apoptosis in Crohn's disease. Previous studies have indicated a beneficial role of herbs-partitioned moxibustion (HPM) in Crohn's disease by decreasing intestinal epithelial apoptosis. However, whether the effect of HPM is through A20 is unclear.

Research motivation

Our findings will suggest a role of HPM in regulating A20 level in anti-apoptotic pathway in the intestinal epithelium of mice with Crohn's disease.

Research objectives

To explore whether HPM alleviates cell apoptosis in the intestinal epithelium by upregulating A20 level in Crohn's disease.

Research methods

Two types of mice were included in this study, namely, mice with A20 deletion in intestinal epithelial cells ($A20^{IEC\text{-}KO}$) and wild-type mice. Both of them were randomly divided into normal control (NC), model control (MC), mesalazine (MESA), and HPM groups. 2,4,6-trinitrobenzene sulfonic acid (TNBS) was administered to establish a Crohn's disease model in the two types. The morphology of the colonic mucosa, serum endotoxin, apoptosis of epithelial cells, protein levels of A20 and tumor necrosis factor receptor (TNFR) 1-related signaling molecules, co-expression of A20 and TNFR1-associated death domain (TRADD), and co-expression of A20 and receptor-interacting protein (RIP) 1 were observed. All data are presented as the mean ± standard deviation.

Research results

Compared with $A20^{IEC\text{-}KO}$ mice, wild-type mice in the HPM group showed that damage of intestinal epithelial barrier was improved, serum endotoxin levels were significantly downregulated ($P < 0.01$), apoptosis percentages were significantly decreased ($P < 0.01$), A20 level was significantly upregulated ($P < 0.01$), and TNFR1, TRADDD, and RIP1 levels were downregulated ($P_{TNF\text{-}a} < 0.01$, $P_{TNFR1} < 0.05$, $P_{TRADD} < 0.05$, $P_{RIP1} < 0.05$). In addition, the co-expression of A20/TRADD and A20/RIP1 showed a predominant yellow fluorescence in WT HPM mice, while a predominantly red fluorescence was noted in $A20^{IEC\text{-}KO}$ HPM mice.

Research conclusions

HPM can upregulate A20 level, resulting in decreased expression of TNFR1, TRADD, and RIP1 to alleviate aberrant cell apoptosis in the intestinal epithelial barrier in Crohn's disease.

Research perspectives

Effect of HPM in decreasing cell apoptosis of intestinal epithelial cells is through upregulating A20 level in Crohn's disease.

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World J Gastroenterol 2019 May 7; 25(17): 2086-2098

DOI: 10.3748/wjg.v25.i17.2086

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Analysis of the autophagy gene expression profile of pancreatic cancer based on autophagy-related protein microtubule-associated protein 1A/1B-light chain 3

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Author contributions: Yang YH, Sun JJ and Fan H designed the research; Zhang YH and Gui Y performed the research; Liu JB contributed novel reagents; Fan H analyzed the data; Yang YH, Zhang YX and Gui Y wrote the paper; Fan H and Yang YH performed critical revision of the manuscript.

Supported by the National Natural Science Foundation of China, No. U1504815 and No. U1504808.

Institutional review board statement: The study was reviewed and approved by the First Affiliated Hospital of Zhengzhou University Institutional Review Board.

Conflict-of-interest statement: The authors declare no competing interests.

Data sharing statement: No additional unpublished data are available.

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Abstract

BACKGROUND

Pancreatic cancer is a highly invasive malignant tumor. Expression levels of the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) and perineural invasion (PNI) are closely related to its occurrence and development. Our previous results showed that the high expression of LC3 was positively correlated with PNI in the patients with pancreatic cancer. In this study, we further searched for differential genes involved in autophagy of pancreatic cancer by gene expression profiling and analyzed their biological functions in pancreatic cancer, which provides a theoretical basis for elucidating the pathophysiological mechanism of autophagy in pancreatic cancer and PNI.

AIM

To identify differentially expressed genes involved in pancreatic cancer autophagy and explore the pathogenesis at the molecular level.

METHODS

Two sets of gene expression profiles of pancreatic cancer/normal tissue

Manuscript source: Unsolicited manuscript

Received: January 9, 2019
Peer-review started: January 10, 2019
First decision: February 26, 2019
Revised: March 20, 2019
Accepted: April 10, 2019
Article in press: April 10, 2019
Published online: May 7, 2019

P-Reviewer: Muhammad JS, Zhang ZY
S-Editor: Ma RY
L-Editor: A
E-Editor: Ma YJ

(GSE16515 and GSE15471) were collected from the Gene Expression Omnibus. Significance analysis of microarrays algorithm was used to screen differentially expressed genes related to pancreatic cancer. Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were used to analyze the functional enrichment of the differentially expressed genes. Protein interaction data containing only differentially expressed genes was downloaded from String database and screened. Module mining was carried out by Cytoscape software and ClusterOne plug-in. The interaction relationship between the modules was analyzed and the pivot nodes between the functional modules were determined according to the information of the functional modules and the data of reliable protein interaction network.

RESULTS

Based on the above two data sets of pancreatic tissue total gene expression, 6098 and 12928 differentially expressed genes were obtained by analysis of genes with higher phenotypic correlation. After extracting the intersection of the two differential gene sets, 4870 genes were determined. GO analysis showed that 14 significant functional items including negative regulation of protein ubiquitination were closely related to autophagy. A total of 986 differentially expressed genes were enriched in these functional items. After eliminating the autophagy related genes of human cancer cells which had been defined, 347 differentially expressed genes were obtained. KEGG pathway analysis showed that the pathways hsa04144 and hsa04020 were related to autophagy. In addition, 65 clustering modules were screened after the protein interaction network was constructed based on String database, and module 32 contains the *LC3* gene, which interacts with multiple autophagy-related genes. Moreover, ubiquitin C acts as a pivot node in functional modules to connect multiple modules related to pancreatic cancer and autophagy.

CONCLUSION

Three hundred and forty-seven genes associated with autophagy in human pancreatic cancer were concentrated, and a key gene ubiquitin C which is closely related to the occurrence of PNI was determined, suggesting that LC3 may influence the PNI and prognosis of pancreatic cancer through ubiquitin C.

Key words: Pancreatic cancer; Autophagy-related protein microtubule-associated protein 1A/1B-light chain 3; Perineural invasion; Gene Ontology analysis; Kyoto Encyclopedia of Genes and Genomes pathway analysis; Ubiquitin C

Core tip: In this study, we identified differentially expressed genes based on the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) to analyze the gene expression profile of autophagy in pancreatic cancer. Three hundred and forty-seven genes that have no confirmed association with the autophagy process of human pancreatic cancer cells in previous studies were concentrated, and the key pathways involved in autophagy were enriched. Furthermore, a key gene ubiquitin C which is closely related to the occurrence of perineural invasion (PNI) was determined, suggesting that LC3 may influence the PNI and prognosis of pancreatic cancer through ubiquitin C.

Citation: Yang YH, Zhang YX, Gui Y, Liu JB, Sun JJ, Fan H. Analysis of the autophagy gene expression profile of pancreatic cancer based on autophagy-related protein microtubule-associated protein 1A/1B-light chain 3. *World J Gastroenterol* 2019; 25(17): 2086-2098
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2086.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2086

INTRODUCTION

Pancreatic cancer is a highly invasive tumor of the digestive system. It is of high

malignancy, its early diagnosis is difficult, and it is not sensitive to radiotherapy and chemotherapy. At present, surgical resection is the only relatively effective treatment. The majority of patients are at the late stage of the disease when diagnosed and thus have missed the best treatment opportunity[1]. Therefore, identifying a new direction for the treatment of pancreatic cancer has become the focus of pancreatic surgery studies. As an important mechanism for tumor cells to escape apoptosis, autophagy has both promoting and inhibiting effects on tumors[2,3]. At present, more and more studies have found that autophagy is closely related to the occurrence, development, differentiation and prognosis of pancreatic cancer[4,5].

Autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3), as a key protein in the autophagy process, is involved in the formation of the autophagosome. A study by our research group found that high expression of LC3 in pancreatic cancer was positively correlated with neural invasion and poor prognosis[6]. On the basis of previous studies and using LC3 as a guidance index, the autophagy gene expression profile of pancreatic cancer was analyzed to guide the functional annotation of differentially expressed genes and to enhance the reliability of bioinformatics prediction and analysis. Differentially expressed genes involved in the autophagy of pancreatic cancer were identified by a gene expression microarray technique. Protein interaction networks were constructed and the functional clustering of differentially expressed genes was carried out. Key interacting proteins or genes between modules were screened and evaluated by statistical methods, and the pathogenesis of pancreatic cancer was explored.

MATERIALS AND METHODS

Sample collection

Two groups of experimental data were selected for the analysis of the whole-genome expression profile. Group 1 consisted of 16 normal pancreatic tissue samples and 36 pancreatic cancer tissue samples. Group 2 consisted of 26 normal pancreatic tissue samples and 26 pancreatic cancer tissue samples. All samples were obtained from surgical excision specimens of pancreatic cancer patients and were diagnosed, classified, graded and staged by pathology professionals.

Gene sequencing

Following the instructions of the Affymetrix gene microarray expression analysis manual and using the Affymetrix Human Genome U133 Plus 2.0 Array sequencing platform (platform number: GPL570)[7-10], gene expression in the samples was examined. All of the obtained data have been uploaded and submitted to the Gene Expression Omnibus (GEO), a gene expression database.

Gene expression profile data acquisition and preprocessing

Based on the information of the above two sets of samples and by exploring the gene expression database of the National Center for Biotechnology Information (NCBI) of the United States[11], two sets of pancreatic tissue gene expression profiles were collected: GSE16515 and GSE15471. Data set-related information is listed in Table 1.

The rma function in the affy package of R language[12] was used to preprocess the raw data of the gene expression profiles, and the robust multi-array average (RMA) algorithm[13] was employed to calculate the amount of gene expression from the raw data of expression profiles and to obtain the gene expression values of the probes, that is, the signal strength values.

Gene annotation of probe data

Using the Annotate package of R language combined with the chip annotation document, the probe sets were labeled with the corresponding genes (Entrez ID). In the situation of a single gene corresponding to multiple probe sets, the average value of the multiple probe sets was used to represent the expression value of the gene. In the case of multiple genes corresponding to one probe set, the probe set data were deleted.

Screening and identification of differentially expressed genes

The significance analysis of microarrays (SAM) algorithm[14] was used to screen for differentially expressed genes related to pancreatic cancer. The analysis platform employed the R language platform.

Functional enrichment analysis of differentially expressed genes

Gene Ontology (GO) analysis of differentially expressed genes was used to search for gene functions whose changes might be correlated with the differentially expressed

Table 1 Relevant information of the genome expression profile data sets of pancreatic tissues

Data set number(raw data of CEL files)	Number of normal samples	Number of disease samples	Platform information
GSE16515	16	36	Affymetrix HG-U133 Plus 2.0, GPL570
GSE15471	26	26	Affymetrix HG-U133 Plus 2.0, GPL570

genes of different samples[15,16]. The pathway analysis of differentially expressed genes was used to search for cellular pathways whose changes might be related to the differentially expressed genes in different samples[17-19]. The significance thresholds of enrichment analysis were 0.01 and 0.05 for GO analysis and pathway analysis, respectively. Descriptions of the result parameters of the functional annotations are shown in Supplementary Table 1.

Protein interaction analysis of differentially expressed genes

Data screening and preprocessing:

Human protein interaction network data were downloaded from the STRING database[20], and protein interaction pairs with interaction scores of more than 900 were selected. Only protein interaction data containing differentially expressed genes were screened from the above qualified protein interaction data. Modules were mined and differentially expressed genes were annotated using the ClusterOne plugin of Cytoscape software.

Analysis of information crosstalk between interacting modules: The calculation method of crosstalk significance is as follows: in the context of a random network, the number of cases in which the number of interaction pairs between modules in N random networks (in this study, N = 1000) was greater than that in real networks was calculated and recorded as n. The formula for calculating the P value is $P = n/N$; a P value less than or equal to 0.05 represents significant crosstalk between modules.

Pivot analysis: The definition of pivot requires satisfaction of the following two conditions: (1) the pivot interacts with two modules at the same time and has at least two interaction pairs with each module; and (2) the P value of the significance analysis of the interaction between the pivot and each module should be less than or equal to 0.05. According to the above descriptions, the Python program was written to find the pivots between the functional modules, and the hypergeometric test method was used for the significance analysis.

RESULTS

Preprocessing results of expression profile raw data

The distribution of gene expression amount calculated by the RMA algorithm is shown in Figure 1A and B After data preprocessing, the gene expression profile data were reduced from the original 54675 probe expression values to 20502 gene expression values.

Extraction results of differentially expressed genes

After data standardization and gene annotation, gene microarray significance analyses were performed on the two sets of data (GSE16515 and GSE15471) separately using the Sam function of the siggenes package of R language (Figure 2A and B); a total of 6098 and 12928 differentially expressed genes were obtained, respectively, and the first 40 genes were selected for display in Supplementary Tables 1 and 2, respectively. A total of 4870 core differentially expressed genes were obtained from the intersection of the two sets of differentially expressed genes for subsequent functional annotation analysis.

Functional enrichment analysis of differentially expressed genes

In the process of GO analysis of differentially expressed genes, the involvement of genes in biological processes, molecular functions and cell compositions was annotated by setting different parameters. The 14 functional items related to apoptosis/autophagy are listed in Table 2.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis enriched the differentially expressed genes into four pathways: hsa03050, hsa04144, hsa05412 and hsa04020. hsa04144 is related to endocytosis, hsa04020 is related to the calcium signaling pathway, and both are, to a certain degree, related to autophagy.

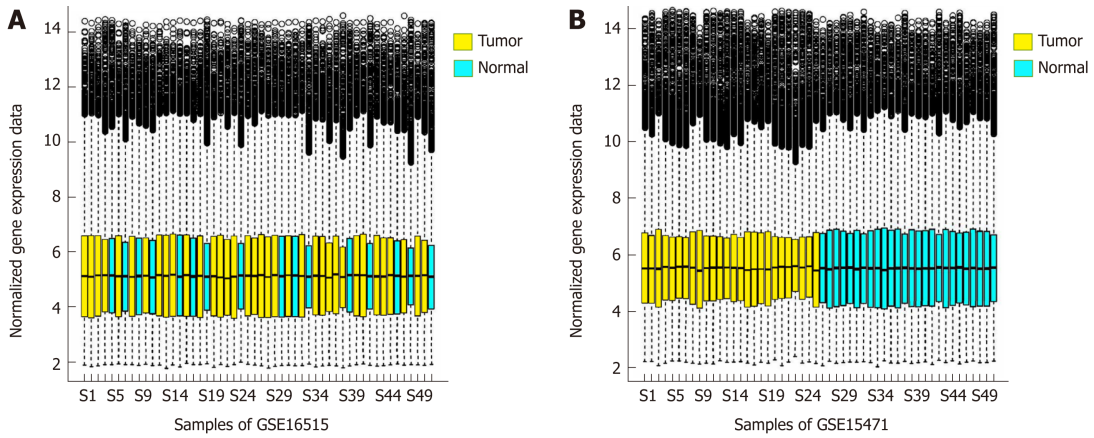

Figure 1 Box diagram of the gene expression distribution. A: Box diagram of the gene expression distribution of each sample after the standardization of set GSE16515. B: Box diagram of the gene expression distribution of each sample after the standardization of set GSE15471.

Searching the GENE functional annotation database of the NCBI using three keywords, autophagy, *Homo sapiens* and cancer (adopt and logic rule), 851 genes were obtained. The 16 functional items associated with autophagy obtained by GO and KEGG analyses revealed a total of 986 differentially expressed genes (Figure 3). After removing genes that are clearly defined in the GENE database, 347 differentially expressed genes were obtained (Supplementary Table 3). The relationship between these genes and the autophagy of pancreatic cancer cells awaits further exploration.

Interaction analysis of differentially expressed genes

Based on a protein interaction network database (STRING), a reliable protein interaction network of 10524 proteins and 196254 interaction pairs was obtained by selecting protein interaction pairs with an interaction score of greater than or equal to 900. Protein interaction data containing only differentially expressed genes were subsequently screened from the above protein interaction data, and a protein interaction network of 2256 differentially expressed genes and 12632 interaction pairs was obtained. Figure 4 shows the protein interaction network composed of differentially expressed genes. Red pivot dots indicate that when mining modules, the *P* values of the participating modules were less than 0.05, while yellow pivot dots indicate that when mining modules, the *P* values of the participating modules were greater than 0.05. Grey pivot dots indicate that these genes were not involved in the modules. A total of 65 clustering modules were screened by importing the above interaction data containing differentially expressed genes into Cytoscape (Supplementary Table 4).

GO and KEGG functional annotation of the differentially expressed genes in the protein interaction network was performed. The results are shown in Supplementary Tables 5 and 6. GO analysis found that GO:0016236 was involved in autophagy, while KEGG analysis revealed a highly significant (P = 1.06E-07) new pathway, hsa04216 (ferroptosis), a new cell death pattern associated with iron death.

Next, we identified the MAP1L3A (LC3) gene in the excavated module 32, which is the target gene of emphasis in this study. We also found that this module contains multiple genes related to autophagy (Supplementary Table 7). Through the functional enrichment analysis of module 32, the genes of this module were found to be mainly involved in pathways related to autophagy and iron-dependent cell death.

Furthermore, network diagrams were used to show the LC3 gene and genes that directly interact with LC3 (Figure 5A) and to demonstrate the functional modules in which the LC3 gene is involved (Figure 5B).

Analysis of crosstalk between interacting modules

The complex intracellular pathway networks were analyzed by crosstalk, and important protein pivots that affect signal transduction between the pathways were identified through protein interactions. The results of the pathway crosstalk analysis of the aforementioned clustering modules are shown in Supplementary Table 8 and Figure 6.

The above functional annotation analysis revealed that modules 33 and 40 were both associated with autophagy; the crosstalk between the two modules is demonstrated using a network diagram by Cytoscape (Figure 7). The genes involved in the two modules and their functional descriptions are listed in Supplementary Table 9.

Table 2 Items related to apoptosis/autophagy in the Gene Ontology enrichment of differentially expressed genes

Category	ID	Description
Biological processes	GO:2001236	Regulation of the extrinsic apoptotic signaling pathway
	GO:0097192	Extrinsic apoptotic signaling pathway in the absence of ligand
	GO:0097191	Extrinsic apoptotic signaling pathway
	GO:2001237	Negative regulation of the extrinsic apoptotic signaling pathway
	GO:0031397	Negative regulation of protein ubiquitination
	GO:0031396	Regulation of protein ubiquitination
	GO:0010038	Response to metal ions
	GO:0045862	Positive regulation of proteolysis
Cell composition	GO:0022624	Proteasome accessory complex
	GO:0034702	Ion channel complex
	GO:0000502	Proteasome complex
	GO:0005838	Proteasome regulatory particle
Molecular functions	GO:0003779	Actin binding
	GO:0002020	Protease binding

Pivot analysis

Pivot analysis revealed that the gene 7316, ubiquitin C (UBC), was associated with several modules related to cancer and autophagy. After removing the redundant modules, seven modules interacting with this pivot were obtained (Supplementary Table 10). These modules all contain genes that directly interact with gene 7316. Functional enrichment analysis showed that most of these 7 modules are related to human cell death, especially the autophagy process. In module 32, the pivot directly interacts with gene 84557 (MAP1LC3A) and is associated with multiple modules (Figure 8).

DISCUSSION

Pancreatic cancer has a high malignant degree, a low early diagnosis rate, and a less than 20% chance of radical resection. The incidence and mortality rate of pancreatic cancer are high. From 2000 to 2014, the overall 5-year survival rate of pancreatic cancer in different regions of the world was between 5% and 15%[21]. In China, approximately 90100 new cases of pancreatic cancer were reported in 2015, ranking ninth among all malignant tumors, while mortality reached 79400 cases, ranking sixth among all malignant tumors; the 5-year survival rate was 9.9%[22]. Until now, an effective diagnosis and treatment for pancreatic cancer have been lacking, and the exact causes and pathogenesis of pancreatic cancer are not well understood. In this study, bioinformatics analysis based on the LC3 gene was used to identify differentially expressed genes in pancreatic cancer and the results were comprehensively analyzed to provide a new experimental basis for the diagnosis and treatment of pancreatic cancer.

First, the GO analysis results of the differentially expressed genes associated with pancreatic cancer were preliminarily studied, and 347 genes that had not been clearly defined by the GENE database as directly related to the autophagy of human cancer cells were identified, providing new information and a novel direction for future cancer and autophagy research. Previous studies have shown that the occurrence and development of pancreatic cancer are the result of the interaction and influence of multiple genes and factors[23,24]. In this study, GO functional annotation analysis revealed that functional annotations were associated with multiple differentially expressed genes, indirectly confirming the earlier results. This study was not limited to an expression abnormality or a mutation of a single gene or protein but rather analyzed the gene microarray expression profile data to obtain the suspected differentially expressed genes associated with tumors, providing useful data for future studies and providing a reliable and detailed experimental basis for the diagnosis and treatment of complex diseases. Second, the KEGG analysis results of pancreatic cancer-related differentially expressed genes were analyzed. The differentially expressed genes were enriched in four pathways, and the complex molecular mechanisms involved in these pathways are related to the occurrence of

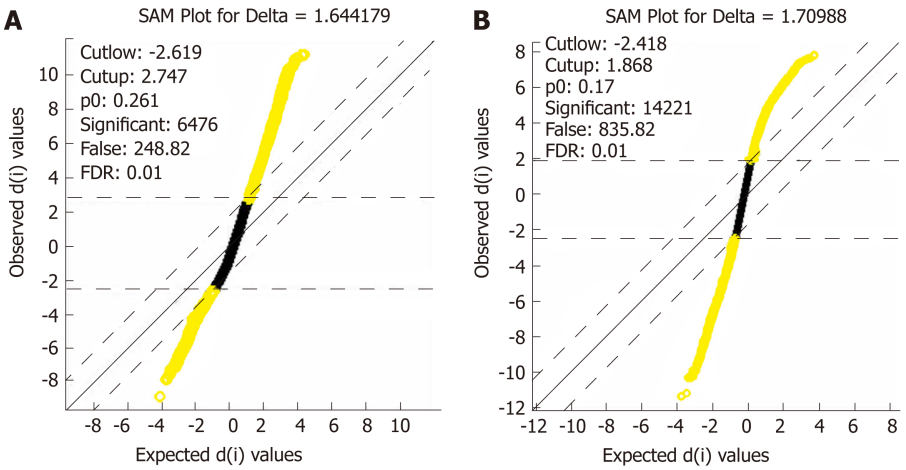

Figure 2 Distribution diagram of the statistical analysis of gene expression. A: Distribution diagram of the statistical analysis of gene expression after the extraction of differentially expressed genes in set GSE16515; B: Distribution diagram of the statistical analysis of gene expression after the extraction of differentially expressed genes in set GSE15471. Yellow for differentially expressed genes and black for non-differentially expressed genes.

tumors, inflammation, immune disorders, and idiopathic diseases[25].

In this study, we combined the gene expression database and the STRING protein interaction database to mine modules in the protein interaction network involving the differentially expressed genes and found that modules 33 and 40 were both associated with autophagy. In view of the functional descriptions, the genes involved in these two modules are closely related to cellular processes such as cancer, autophagy and apoptosis. Through functional enrichment analysis of the genes involved in these modules, KEGG enrichment further identified pathway hsa04216, that is, ferroptosis[26], which is characterized by the production of reactive oxygen species (ROS) from the peroxidation of accumulated iron and lipids. Kang *et al*[27] reviewed the relationship between autophagy and ferroptosis and noted that the activation of ferroptosis depends on its induction by autophagy.

In the module interaction study, we found that UBC, as a pivot of module interactions, connected several modules related to cancer and autophagy and plays an important role in multiple cell death modules related to autophagy (Figures 1-8), consistent with current knowledge and our understanding of cancer and cell death. UBC, as a precursor of ubiquitin, plays a key role in the occurrence and development of diseases such as autophagy, cancer, and inflammation through the ubiquitin proteasome system (UPS)[28]. A large number of studies have shown a close relationship between UPS and autophagy[29,30]. Previously, UPS and autophagy have been considered complementary degradation systems with no intersection. However, some monoubiquitinated proteins can also be degraded by autophagy[31]. Pandey *et al*[32] performed in vitro experiments and showed that monoubiquitination and histone deacetylase 6 (HDAC6) are the key signals linking the two systems of autophagy and UPS, consistent with the results of the current study that the ubiquitin precursor UBC connects multiple autophagy-related modules.

A previous study showed that the signaling pathway adapter protein P62 contains an LC3 recognition sequence that forms oligomers to recruit ubiquitinated proteins through a ubiquitin binding domain and interacts with LC3 to form degradation substrates for the autophagosome[33]. Ubiquitin is also one of the substrates of molecular chaperone-mediated autophagy[34]. In this study, a direct interaction between LC3 and the pivot gene UBC was identified, which has certain significance for the study of the mechanism by which autophagy scavenges ubiquitinated substrates. Ubiquitin and UPS are also closely related to the occurrence and development of tumors. Tang *et al*[35] confirmed that ubiquitin is highly expressed in many types of tumor tissues. Liu *et al*[36] showed that UPS could selectively degrade the products of oncogenes and tumor suppressor genes, as well as apoptosis-regulating proteins, thus regulating cell mutation and tumorigenesis.

In recent years, the perineural invasion (PNI) of pancreatic cancer has become a research hotspot in academia and in the clinic; however, its mechanism has not yet been elucidated. A complicated tumor microenvironment, autophagy, and neural plasticity[37] exert important effects on the nerves around and inside the pancreas. It has been reported[38] that pancreatic cancer tissue with high expression of ubiquitin-specific protease 9X (USP9X), a member of the ubiquitin-specific protease subfamily

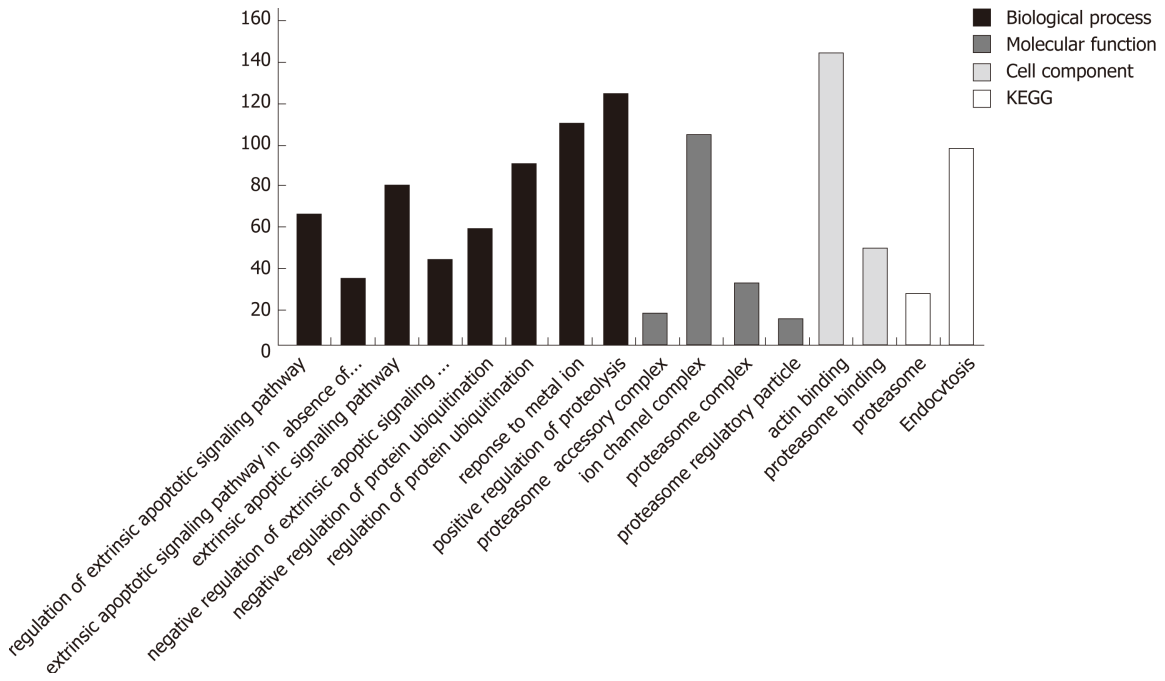

Figure 3 Distribution of the numbers of differentially expressed genes in functional items associated with apoptosis/autophagy.

of the family of deubiquitinating enzymes, may be more invasive, and high expression of USP9X is closely associated with the prognosis of pancreatic cancer. In UPS, the reversible process of protein ubiquitination and deubiquitination catalyzed by ubiquitinating enzymes (UBEs) and deubiquitinating enzymes (DUBs) is also closely related to PNI of the tumor[39]. UBC-terminal hydrolases (UCHs) are a subfamily of DUBs. Previous studies have shown that the UCH family plays different roles in the progression of different tumors[40]. Ubiquitin carboxyl terminal esterase L1, a family member of UCHs, is not only overexpressed in neural tissue[41] but is also used as a marker of nerve fibers to study PNI[42].

In the study of modular clustering based on protein interactions, UBC was clustered with multiple autophagy-related genes in module 32 and directly interacted with LC3 and NBR1 (Figures 2-7). UCH uses ubiquitinated proteins as substrates to catalyze the removal of ubiquitin molecules. Therefore, UBC and UCH also directly interact. Moreover, the expression level of UCH is closely related to the process of PNI. Based on our previous study[6] in which the positive rate of PNI was significantly positively correlated with the high expression of LC3 in pancreatic cancer patients and that PNI and LC3 levels are independent risk factors for the poor prognosis of pancreatic cancer, we postulate that the autophagy-related protein LC3 may establish a close association with pancreatic cancer PNI through UBC and its associated ubiquitin proteasome system.

In summary, the present study combined the gene expression profile microarray technique with bioinformatics analysis technology to analyze and mine a large quantity of data and identified new significantly differentially expressed genes related to the occurrence of autophagy in pancreatic cancer, which expands the autophagy-related gene profile of pancreatic cancer and is helpful for the search of candidate susceptible genes and rare mutations that may be associated with the occurrence and development of autophagy in pancreatic cancer. The identification and review of UBC, a key gene that directly interacts with LC3, suggest that it may be a key factor that leads to a poor prognosis of pancreatic cancer mediated by PNI and suggests a new direction for further research.

In this study, we identified differentially expressed genes between pancreatic cancer cells and normal cells at the whole-genome level using the whole-genome expression profiling technique, identified 347 genes that have no confirmed association with the autophagy process of human pancreatic cancer cells in previous studies, and discovered and clarified information about the pathways involved in autophagy. Furthermore, we identified UBC, which plays an important role in several modules related to cell death, through gene expression microarray analysis based on autophagy and LC3 and found that UBC is widely involved in tumor cell growth, invasion and metastasis. It was also found that ubiquitin is closely related to PNI. It is believed that LC3 may affect the PNI and prognosis of pancreatic cancer through

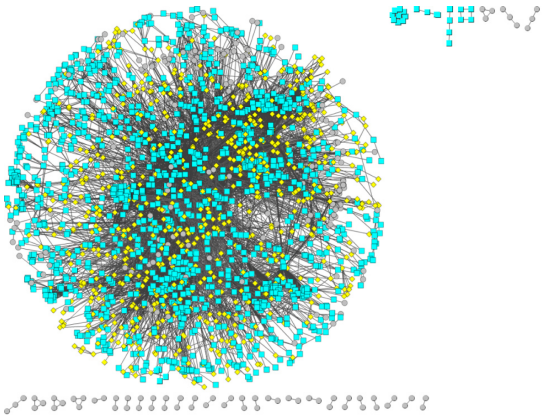

Figure 4 Protein interaction network composed of differentially expressed genes.

UBC, which is helpful for the study and treatment of pancreatic cancer and provides an important clue to the pathogenesis of pancreatic cancer.

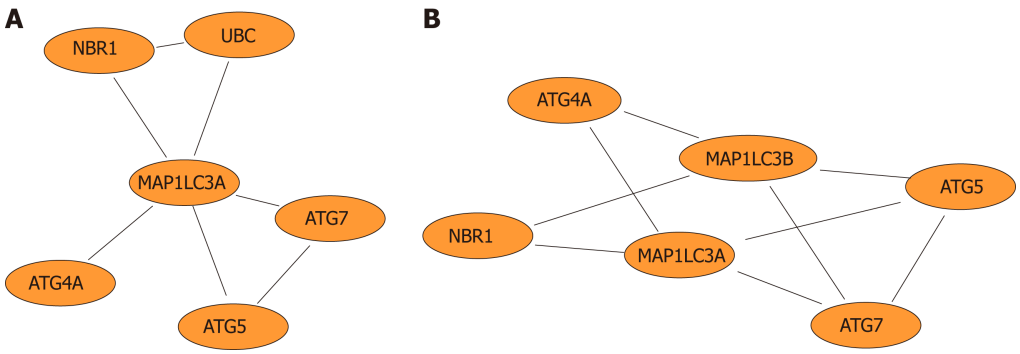

Figure 5 Network diagram. A: Network diagram of genes that directly interact with the autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3) gene; B: Network diagram of functional modules in which the LC3 gene is involved.

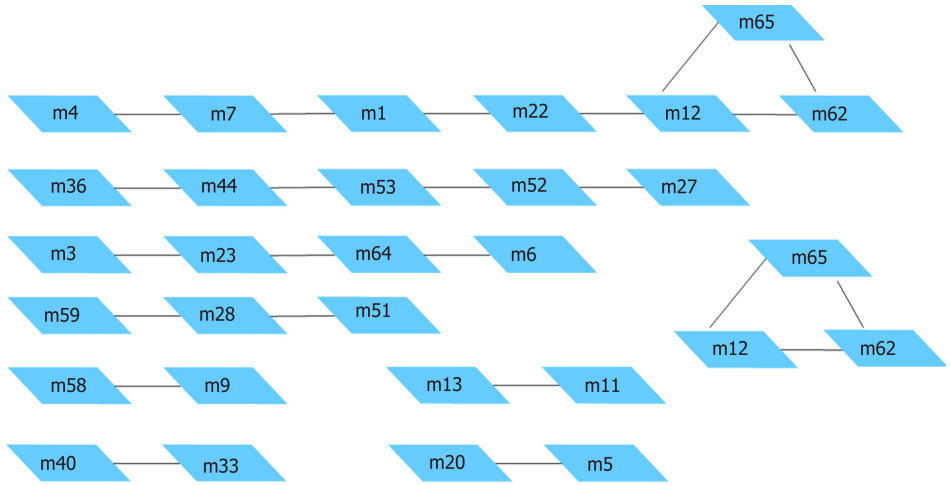

Figure 6 Module pairs with significant crosstalk.

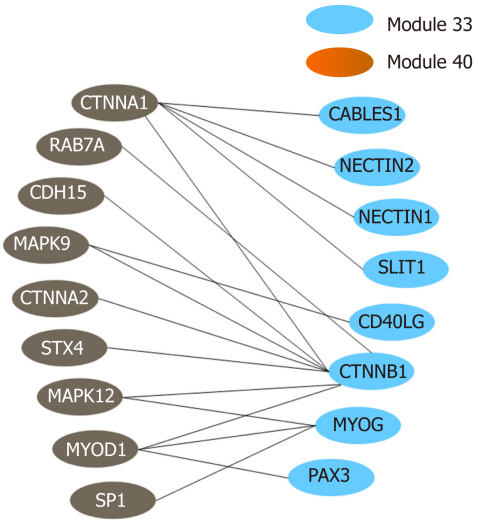

Figure 7 Crosstalk relationship between modules 33 and 44.

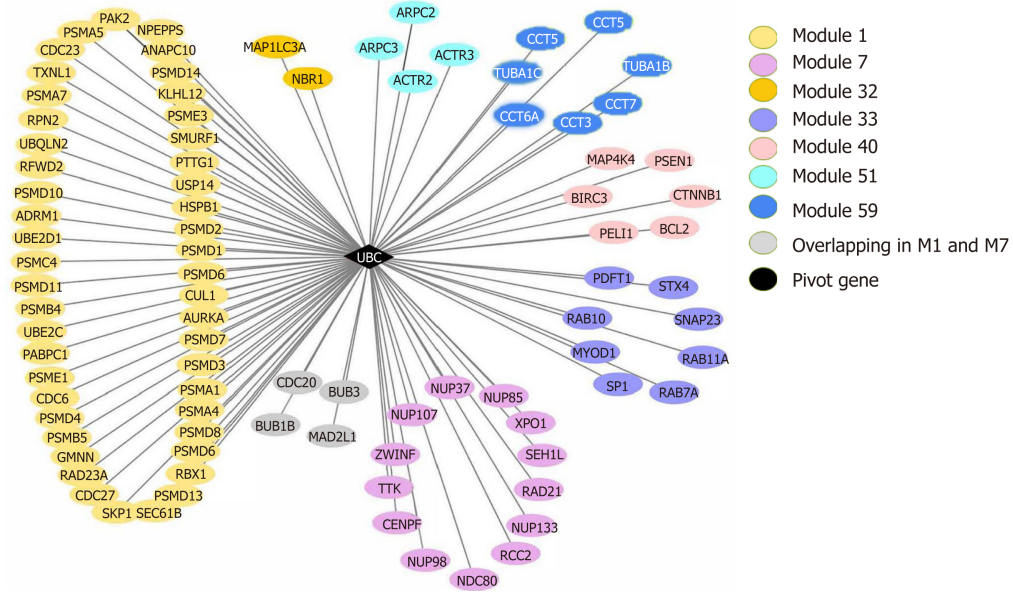

Figure 8 The ubiquitin C gene (pivot) and its connecting modules.

ARTICLE HIGHLIGHTS

Research background

Pancreatic cancer is a malignant tumor with a poor prognosis that has almost equal mortality and morbidity in patients. At present, more and more studies have found that autophagy is closely related to the occurrence, development, differentiation and prognosis of pancreatic cancer. Autophagy-related protein microtubule-associated protein 1A/1B-light chain 3 (LC3), as a key protein in the autophagy process, is involved in the formation of the autophagosome. A study by our research group found that high expression of LC3 in pancreatic cancer was positively correlated with neural invasion and poor prognosis. With the development of genomics, gene expression microarray technology, proteomics and bioinformatics, the ability to manipulate and characterize human genes and their products has been acquired. Disease-related genes have been studied at the molecular level to understand the pathogenesis of diseases. On the basis of previous studies and using LC3 as a guidance index, the autophagy gene expression profile of pancreatic cancer was analyzed to guide the functional annotation of differentially expressed genes and to enhance the reliability of bioinformatics prediction and analysis. Thus, to provide a basis for the study of the molecular mechanism of autophagy in pancreatic cancer.

Research motivation

This study focused on the differentially expressed genes based on LC3 to analyze the gene expression profile of autophagy in pancreatic cancer. Thus, to provide a basis for the study of the molecular mechanism of autophagy in pancreatic cancer.

Research objectives

To identify differentially expressed genes in autophagy of pancreatic cancer and to provide a basis for exploring the molecular mechanism of autophagy of pancreatic cancer cells and finding new targets for diagnosis and treatment of pancreatic cancer.

Research methods

On the basis of previous studies and using LC3 as a guidance index, differentially expressed genes involved in the autophagy of pancreatic cancer were identified by a gene expression microarray technique. Protein interaction networks were constructed and the functional clustering of differentially expressed genes was carried out. Key interacting proteins or genes between modules were screened and evaluated by statistical methods, and the pathogenesis of pancreatic cancer was explored.

Research results

After removing genes that are clearly defined in the GENE database, 347 differentially expressed genes were obtained. The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed a highly significant new pathway, hsa04216 (ferroptosis), a new cell death pattern associated with iron death. The ubiquitin C (UBC) as a pivot of module interactions, connected several modules related to cancer and autophagy and plays an important role in multiple cell death modules related to autophagy.

Research conclusions

In this study, we identified differentially expressed genes based on the LC3 to analyze the gene

expression profile of autophagy in pancreatic cancer. Three hundred and forty-seven genes that have no confirmed association with the autophagy process of human pancreatic cancer cells in previous studies were concentrated, and the key pathways involved in autophagy were enriched. Furthermore, a key gene UBC which is closely related to the occurrence of perineural invasion (PNI) was determined, suggesting that LC3 may influence the PNI and prognosis of pancreatic cancer through UBC.

Research perspectives

With the development of genomics, gene expression microarray technology, proteomics and bioinformatics, we have been able to study disease-related genes at the molecular level to understand the pathogenesis of disease, thus to seek new research directions or to find new targets for clinical diagnosis and treatment. In this study, LC3 was used as a target to explore the differential genes related to autophagy in pancreatic cancer cells. Three hundred and forty-seven genes that have no confirmed association with the autophagy process of human pancreatic cancer cells in previous studies were concentrated, it is obviously unrealistic to analyze all the genes interacting with LC3 *in vitro*. Nevertheless, a key gene UBC which is closely related to the occurrence of PNI was determined. Our previous results showed that the high expression of LC3 was positively correlated with PNI in the patients with pancreatic cancer. Suggesting that LC3 may influence the PNI and prognosis of pancreatic cancer through UBC. Therefore, we have planned to supplement some vitro and vivo experiments to further analysis the relationship between them and explore the molecular mechanism of phagocytosis in pancreatic cancer cells.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 May 7; 25(17): 2099-2109

DOI: 10.3748/wjg.v25.i17.2099

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Retrospective Study

Clinical value of preoperative methylated septin 9 in Chinese colorectal cancer patients

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Author contributions: Yang X and Xu ZJ designed the research scheme and contributed equally to this article; Yan YL and Gong ZC contributed equally to correspondence about this manuscript; Chen X, Zeng SS, Qian L, and Wei J collected data from the samples; Peng M, Wang X, Liu WL, Xu ZJ, and Ma HY analyzed the data.

Supported by the National Natural Science Foundation of China, No. 81803035, No. 81703036, and No. 81572946; the China Postdoctoral Science Foundation, No. 2017M610510; and the Youth Fund of Xiangya Hospital, No. 2017Q17.

Institutional review board statement: This study was reviewed and approved by the Ethical Committee of Xiangya Hospital of Central South University (Approval No. 2018111100).

Informed consent statement: According to the "Human Biomedical Research Ethical

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Abstract

BACKGROUND

The methylated septin 9 (mSEPT9) assay was the first blood-based test approved by the United States Food and Drug Administration as a colorectal screening test. However, the diagnostic and prognostic role of preoperative mSEPT9 for colorectal cancer (CRC) in Chinese patients is still unknown.

AIM

To improve the understanding of diagnostic and prognostic factors, serum mSEPT9 was detected in Chinese CRC patients.

METHODS

A retrospective analysis of 354 cases, of which 300 had CRC and 54 were normal, was performed in China. Patients' characteristics, treatments, and laboratory data, including age, the date of surgery, Union for International Cancer Control (UICC) stages, distant metastasis (M), and so on, were collected. Methylation levels of SEPT9 were quantified by quantitative, methylation-specific polymerase chain reaction before surgery. In addition, the effects of mSEPT9 on the occurrence and prognosis of 330 CRC cases from The Cancer Genome Atlas (TCGA) database were evaluated using bioinformatics analyses. Potential prognostic factors for overall survival (OS) and progression-free survival (PFS) were evaluated by Kaplan-Meier univariate analysis.

RESULTS

In Chinese CRC patients, positive mSEPT9 was strongly associated with advanced UICC stages, deeper invasion by the primary tumor, and more distant metastasis. Methylation levels of SEPT9 were stage-dependent and showed a stepwise increase in UICC stages (I–IV), primary tumor categories (T1–T4), regional node categories (N0–N2), and distant metastasis categories (M0–M1). The patients with positive mSEPT9 showed a tendency toward lower PFS. After analyzing TCGA clinical data, the high mSEPT9 group was found to be obviously correlated only with more distant metastasis. The patients with high mSEPT9 levels showed a tendency toward lower OS. Besides, nine meaningful mSEPT9 sites were found to provide guidance for the follow-up studies.

CONCLUSION

MSEPT9 analysis may add valuable information to current tumor staging. Serum mSEPT9 in Chinese CRC patients appears to offer promising novel prognostic markers and might be considered for monitoring CRC recurrence.

Key words: Methylated septin 9; Methylated; Colorectal cancer; Diagnosis; Prognosis

Core tip: This study retrospectively explored the value of serum septin 9 methylation (mSEPT9) in the diagnosis and prognosis of colorectal cancer in a Chinese population. Preoperative mSEPT9 levels in 354 enrolled patients were retrospectively analyzed. In addition, the effects of mSEPT9 on the occurrence and prognosis of 330 colorectal cancer cases from The Cancer Genome Atlas database were evaluated using bioinformatics analyses. Besides, nine meaningful mSEPT9 sites were found to provide guidance for the follow-up studies.

Citation: Yang X, Xu ZJ, Chen X, Zeng SS, Qian L, Wei J, Peng M, Wang X, Liu WL, Ma HY, Gong ZC, Yan YL. Clinical value of preoperative methylated septin 9 in Chinese colorectal cancer patients. *World J Gastroenterol* 2019; 25(17): 2099-2109
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2099.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2099

INTRODUCTION

Colorectal cancer (CRC) remains the third most common cancer expected to occur in men and women[1], accounting for approximately 10% of the global cancer burden. To date, more than 90% of patients with early CRC have survived five years after diagnosis[2,3]. However, in the case of regional spread to lymph nodes or adjacent organs, the five-year relative survival rate decreases to 69%, and when there is distant metastasis, it drops sharply to approximately 10%[3,4]. Despite significant recent achievements in the diagnosis and treatment of these patients, resulting in partial reductions in overall incidence and mortality, there is no effective diagnostic assay so far for tumor progression or recurrence monitoring, especially *in vitro*.

Detection of CRC recurrences or metastases in the early stage by constant monitoring may improve long-term outcomes through timely treatment. The American Joint Committee on Cancer (AJCC) Cancer Staging, seventh edition has accepted clinically useful carcinoembryonic antigen (CEA) serum tumor marker as a site-specific prognostic factor in CRC[5]. However, the low detection sensitivity of CEA hinders its use for many surgical patients, because patients with negative CEA results before surgery usually cannot be monitored after surgery[6,7]. In addition, periodic computed tomography (CT) scanning is another noninvasive method for surgical therapeutic effect assessment[8]. However, CT scans have limited sensitivity and high false positive rates, and cannot be used routinely as a monitoring examination due to the danger of long-term radiation[9]. Therefore, development of novel, sensitive biomarkers for monitoring recurrences or metastases of CRC is urgently needed.

Hypermethylation of the promoter of septin 9 (SEPT9) has previously been shown to be a sensitive and specific biomarker in various cancers including CRC[10-13] and its precursor lesions[14-16]. As a result, the methylated SEPT9 (mSEPT9) assay became the first blood-based test approved by the United States Food and Drug Administration

Review Procedures" approved by the National Health and Family Planning Committee of China (No. 11, Section 39), informed consent was waived because of the retrospective nature of the study. After the following circumstances have been reviewed and approved by the ethics committee, the informed consent form can be waived if: Research is conducted using human body materials or data that can identify information, and the subjects can't be found, and the research project does not involve personal privacy and commercial interests.

Conflict-of-interest statement: The authors have no conflicts of interest to disclose.

Manuscript source: Unsolicited manuscript

Received: February 14, 2019
Peer-review started: February 15, 2019
First decision: March 5, 2019
Revised: March 25, 2019
Accepted: April 10, 2019
Article in press: April 10, 2019
Published online: May 7, 2019

P-Reviewer: Matsui T, Vetvicka V
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

as a CRC screening test[17]. A study of Korean CRC patients found that serum mSEPT9 had a tendency to show metastasis and a low disease-free survival rate[18]. In a recent study of German CRC patients, mSEPT9 was significantly associated with Union for International Cancer Control (UICC) stages both before and after therapy[19]. In addition, quantitative mSEPT9 levels have been successfully applied for the diagnosis of CRC[19-22], and for the screening, diagnosis, monitoring, prognosis, and molecular staging of head and neck squamous cell carcinomas (HNSCC)[19]. However, the diagnostic and prognostic role of preoperative mSEPT9 for CRC in Chinese patients is still unknown.

This study assessed the correlation between clinicopathological characteristics and preoperative serum mSEPT9 in Chinese CRC patients and, further, to confirm the correlation between mSEPT9 levels and CRC prognosis by bioinformatics analyses. In addition, we analyzed methylated sites that were co-upregulated or codownregulated in colon and rectum tumors, to provide the theoretical guidance for further research.

MATERIALS AND METHODS

Patients and samples

This present study was conducted from December 2017 to November 2018 among patients at the Department of Hepatobiliary and Enteric Surgery in Xiangya Hospital. A total of 354 subjects with mSEPT9 serum detection before surgery were recruited from a medicine-pharmacy-nursing integrative parenteral medication rational use and safety early warning platform, the Parenteral Prescription Early Warning and Assessment System, including 300 CRC patients and 54 normal subjects. This study was approved by the Ethical Committee of Xiangya Hospital of Central South University (Approval No. 2018111100).

Three hundred patients presented with histologically confirmed primary CRC. Recurrences or metastases were determined from diagnostic tests (CT scan, magnetic resonance imaging, or colonoscopy) and confirmed through tissue pathology when available[7]. Clinical parameters, including mSEPT9 detection results, gender, age, UICC stage, histologic grade, primary tumor (T) categories, regional node (N) categories, distant metastasis categories (M), lymphatic invasion (L), lymph nodal status, vascular invasion (V), and tumor site, were collected. The UICC stage, tumor node metastasis (TNM) categories and histologic differentiation were graded on the basis of the eighth edition of the AJCC[23]. Progression-free survival (PFS) time was calculated from the CRC patients' date of surgery to presentation of clinical or pathological evidence of cancer recurrence.

In addition, 330 colorectal adenocarcinomas from The Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov/.) were selected and analyzed retrospectively. Patients whose mSEPT9 levels were less than or equal to median were assigned to the low mSEPT9 group, whereas others were assigned to the high mSEPT9 group. The overall survival (OS) time was calculated from the CRC patients' date of surgery to the date of dead or to the last contact date.

Methylated SEPT9 detection

A 10 mL peripheral blood sample was collected with a 10 mL K2EDTA anticoagulant tube for the SEPT9 assay [BioChain (Beijing) Science and Technology, Inc., Beijing, China]. Peripheral blood sample storage and transportation, DNA extraction, and bisulfite conversion were performed manually following the manufacturer's instructions of the Epi proColon 2.0 kit (Epigenomics AG, Berlin, Germany). The mSEPT9 was assayed with the Epi proColon 2.0 kit on an AB7500 Fast Dx Real Time polymerase chain reaction device (Life Technologies) in the Clinical Laboratory of Xiangya Hospital, Central South University. Briefly, a polymerase chain reaction (PCR) test was performed in triplicate with 15 μL template DNA per well and run for 45 cycles[24]. The instrument software was used to record the PCR results for β-actin (ACTB) and methylated SEPT9 from each of the triplicate reactions. The validity of each sample batch was determined according to methylated SEPT9 and ACTB threshold count (Ct) values for the positive and negative controls. ACTB served as an internal reference to assess the integrity of each sample. According to the instructions, Ct value was less than 41.1 was assigned to the positive mSEPT9 group, whereas those whose Ct value was over 41.1 were assigned to the negative mSEPT9 group.

Statistical analyses

All statistical analyses were performed using SPSS 18 software (SPSS Inc, Chicago, United States). The measurable data was expressed as the mean and standard deviation (SD). Differences of clinicopathological characteristics and Ct values

between groups were compared *via* *t*-tests and $\chi 2$ test. The univariate analysis was performed to assess the effect of mSEPT9 to predict PFS and OS by the Kaplan-Meier method. Binary logistic regression was used to analyze the association between each genetic biomarker (*e.g.*, mismatched repair proteins) and mSEPT9. All the statistical tests were bilateral, and $P < 0.05$ was considered statistically significant.

RESULTS

The mSEPT9 in CRC patients and normal subjects

Among Chinese CRC patients, the methylated Ct values of 300 primary CRC patients and 54 normal subjects were analyzed. Based on contradictory trends for Ct value and expression, the preoperative serum mSEPT9 levels were significantly higher in CRC patients than in the normal subjects ($P = 0.008$) (Figure 1A). The positive rate of mSEPT9 was 52.3% for CRC patients and 25.9% for normal subjects ($P = 0.102$) (Figure 1B).

Among 351 patients from the TCGA database, the mSEPT9 levels of 330 CRC patients and 21 normal subjects were analyzed. The serum mSEPT9 levels of the CRC patients were higher than those of the normal subjects, but were not statistically significant ($P = 0.530$) (Supplemental Figure 1).

Clinicopathological characteristics and mSEPT9 in CRC

The Chinese CRC patients, clinicopathological features of 300 CRC patients are described in Table 1. As shown in Figure 1C, patients older than 50 years were statistically more numerous than those aged 50 or younger in both positive and negative groups ($P = 0.016$). Through analyzing UICC stages, we found that the positive rate of stage III was observably higher than stage I (46.6% *vs* 31.0%, $P = 0.012$) (Figure 2A); mSEPT9 levels showed a significant increase from UICC stages II to III ($P = 0.033$) and stages III to IV ($P < 0.0001$), but no obvious difference was detected between stages I to II ($P = 0.898$, Figure 3A).

In addition, the association of mSEPT9 levels and rate of positive mSEPT9 among primary tumor categories (T1–T4), regional node categories (N0–N2) and distant metastasis categories (M0–M1) were also analyzed. The detection rate of positive T3 was observably higher than that of T1 (51.1% *vs* 40.0%, $P = 0.019$) (Figure 2B). Positive rate and levels of mSEPT9 revealed a significant increase from T3 to T4 ($P = 0.030$, $P = 0.046$, respectively) (Figure 2B, Figure 3B). In terms of regional node categories, N0 to N2 showed a gradual increase in mSEPT9 levels ($P = 0.012$) (Figure 3C), but did not show any association with the rate of positive mSEPT9 (Figure 2C). As shown in Figures 2D and 3D, mSEPT9 showed the best ability to discriminate between local and metastatic CRC ($P = 0.015$, $P < 0.0001$, respectively). However, higher mSEPT9 levels were not found in CRC patients with lymphatic or vascular invasion than in those without invasion (all $P > 0.05$). We also failed to find association among MLH1, MSH2 (25D12), MSH6, PMS2, and Ki67 and mSEPT9 (all $P > 0.05$) (Supplemental Table 1).

The clinicopathological features of 330 CRC patients from the TCGA database are described in detail in Supplemental Table 2. Similarly, there was a tendency for more distant metastasis ($P = 0.0001$) and more CRC patients older than 50 ($P < 0.0001$) in the high mSEPT9 group, but no significant difference was found in UICC stages, primary tumor categories, or regional node categories (all $P > 0.05$).

Prognostic significance of mSEPT9 in CRC patients

Kaplan-Meier univariate analysis showed that positive mSEPT9 was obviously associated with shorter PFS among the Chinese CRC patients ($P = 0.019$, Figure 4A). The positive mSEPT9 CRC cases were estimated to have an mean PFS duration of 3.7 mo [95% confidence interval (CI): 2.14-5.19] compared with the 6.0 mo (95%CI: 0-13.87) in the negative mSEPT9 CRC cases.

In addition, serum mSEPT9 showed prognostic significance for the CRC patients from the TCGA database ($P = 0.008$, Figure 4B). CRC patients with low mSEPT9 levels were found to be correlated with longer OS. The low mSEPT9 CRC cases had an estimated mean OS duration of 8.1 months (95%CI: 6.53-9.27) compared with the 5.1 mo (95%CI: 3.87-6.33) in the high mSEPT9 CRC cases.

Significant methylation sites for SEPT9

In further analyzed TCGA clinical data, 124 mSEPT9 sites were found that showed differential expression among normal subjects and those with colon and rectum adenocarcinoma, respectively (all $P < 0.05$) (Supplemental Figure 2). After analyzing the detailed information of these 124 mSEPT9 sites, 68 co-upregulated and 36 co-downregulated mSEPT9 sites in CRC adenocarcinoma were further observed. We finally confirmed that there were eight co-upregulated mSEPT9 sites (Figure 5) and

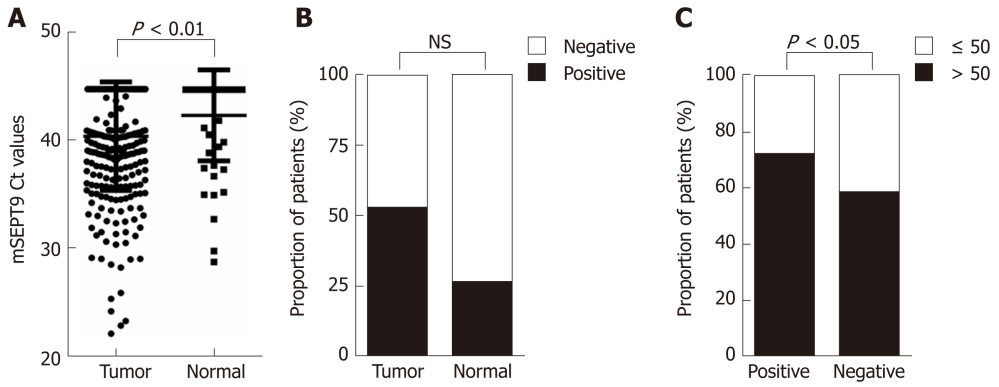


Figure 1 Graphical representations of difference in methylated septin 9 Ct values or proportion of patients between different groups. A: Methylated septin 9 (mSEPT9) Ct values in tumor group and normal group; B: Proportion of patients with positive and negative mSEPT9 in tumor group and normal group; C: Proportion of patients older than 50 and aged 50 or younger in positive group and negative group. The statistical significance for difference of means is shown in P values, *t*-test, or $\chi 2$ test. mSEPT9: Methylated septin 9.

one co-downregulated mSEPT9 site (cg02975107) through setting a cut-off of a two-fold expression change of mSEPT9.

DISCUSSION

Most patients with early CRC undergo curatively intended surgery to clear up primary lesions and local lymph node metastasis up. However, 30%-50% of patients would still confront tumor recurrence and might die from metastasis[25]. Timely monitoring of recurrence and metastasis is of great significance to the prognosis and survival of patients. In our study, mSEPT9 was proved to be an effective bio-marker for diagnosis, recurrence, and prognosis of CRC in Chinese patients, and nine significant mSEPT9 sites were confirmed for further in-depth consideration.

Our study confirmed the value of serum mSEPT9 for CRC diagnosis. Compared with normal tissues in Chinese and TCGA data, serum SEPT9 was found to be hypermethylated in tumor tissues, which was consistent with previous studies[18,19,26]. Studies showed that age affected the detection rate of the SEPT9 assay[27,28], and we found that a positive rate of mSEPT9 was strongly associated with CRC patients aged over 50 years both in Chinese and TCGA data. This accords with the definition of an average risk population in National Comprehensive Cancer Network Guidelines for CRC[29]. Remarkably, we reported that SEPT9 performs outstandingly as an auxiliary molecular staging parameter in the Chinese population, especially because mSEPT9 levels could distinguish between pathological UICC and TNM stages in an incremental fashion. In addition, our data demonstrated that CRC patients in earlier tumor stages showed lower mSEPT9 levels compared to those with more advanced lesions, which is consistent with studies in German CRC patients[19,30-32]. Most importantly, its ability to identify patients with distant metastases emphasizes the potential of mSEPT9 as a bio-marker, which adds valuable information to the classification of tumors[33-35]. However, high mSEPT9 group did not show any association with UICC, T, or N stages in patients from the TGGA database, who were from American Indian, Asian, Black, or African American populations. This might be explained by the different study populations. Previous studies found that the incidence of CRC and the sensitivity to the mSEPT9 test assay in different ethnic groups were different[14,36].

In addition, serum mSEPT9 were proved to be an independent predictors of CRC recurrence and unfavorable cancer-specific survival in Chinese and TCGA data, which is consistent with previous studies in Singapore and Germany[19,37,38]. The study was performed with a large number of prognostic features and patients; however, much longer prognosis and follow-up time are necessary before final conclusions can be made, and the increasing number of patients with earlier-stage CRC demands a widening of the clinical importance of predictive value for prognosis.

After further analysis of the TCGA clinical data, we obtained nine SEPT9 methylation sites that show two-fold higher or lower mSEPT9 levels in CRC than normal tissues. However, no studies were found at present that investigate the prognosis of these methylation sites and CRC was found. Surprisingly, cg12783819, which only shows 1.5-fold higher mSEPT9 levels in CRC than in normal tissues, has been proven to be able to assess the diagnosis, prognosis, and molecular staging of

Table 1 Clinicopathological characteristics based on methylated septin 9 status in 300 colorectal cancer patients

Parameter		Positive group (%)	Negative group (%)	*P*
Gender	Male	84 (28.0)	81 (27.0)	0.585
	Female	73 (24.3)	62 (20.7)	
Age	≤ 50 yr	45 (15.0)	60 (20.0)	0.016
	> 50 yr	112 (37.3)	83 (27.7)	
UICC stage	I	9 (3.0)	20 (6.7)	0.020
	II	46 (15.3)	32 (10.7)	
	III	62 (20.6)	71 (23.7)	
	IV	13 (4.3)	6 (2.0)	
	Unknown	27 (9.0)	14 (4.7)	
Histologic grade	Low level	115 (38.3)	103 (34.3)	0.972
	High level	18 (6.0)	17 (5.7)	
	Not recorded	24 (8.0)	23 (7.7)	
Primary tumor (T) category	T1	2 (0. 7)	3 (1.0)	0.002
	T2	15 (5.0)	23 (7.7)	
	T3	70 (23.3)	67 (22.3)	
	T4	40 (13.3)	34 (11.3)	
	Not recorded	30 (10.0)	16 (5.3)	
Regional node (N) category	N0	57 (19.0)	23 (7.7)	0.852
	N1	32 (10.7)	67 (22.3)	
	N2	37 (12.3)	34 (11.3)	
	Not recorded	31 (10.3)	16 (5.3)	
Distant metastasis (M)	Absent	141 (47.0)	137 (45.7)	0.015
	Present	16 (5.3)	6 (2.0)	
Lymphatic invasion (L)	Absent	58 (19.3)	60 (20.0)	0.217
	Present	51 (17.0)	52 (17.3)	
	Not recorded	48 (16.0)	31 (10.3)	
Lymph nodal status	No node involved	51 (17.0)	52 (17.3)	0.048
	1-3 lymph node involved	27 (9.0)	40 (13.3)	
	> 4 lymph node involved	31 (10.3)	20 (6.7)	
	Not recorded	48 (16)	31 (10.3)	
Vascular invasion (V)	Absent	36 (12.0)	31 (10.3)	0.278
	Present	70 (23.3)	76 (25.3)	
	Not recorded	51 (17.0)	36 (12.0)	
Tumor site[a]	Left colon	44 (14.7)	31 (10.3)	0.022
	Right colon	27 (9.0)	11 (3.7)	
	Rectum	74 (24.7)	86 (28.6)	
	Unable to distinguish	12 (4.0)	15 (5.0)	

[a]Right colon includes cecum through transverse colon, whereas left colon includes splenic flexure, descending colon, and sigmoid colon. Clinicopathological characteristics between positive group and negative group were analyzed using $\chi 2$ test. UICC: Union for International Cancer Control.

German HNSCC and CRC patients[19,20]. The result prompts us to explore the potential association between these nine methylation sites and in Chinese CRC patients in the future.

In conclusions, serum SEPT9 methylation testing is a powerful additional diagnostic tool and promising, novel prognostic markers. Patients with initially high mSEPT9 levels may benefit from intensive therapy and close monitoring of disease development, thereby improving outcomes for CRC patients. These patients may benefit from early systemic treatment.

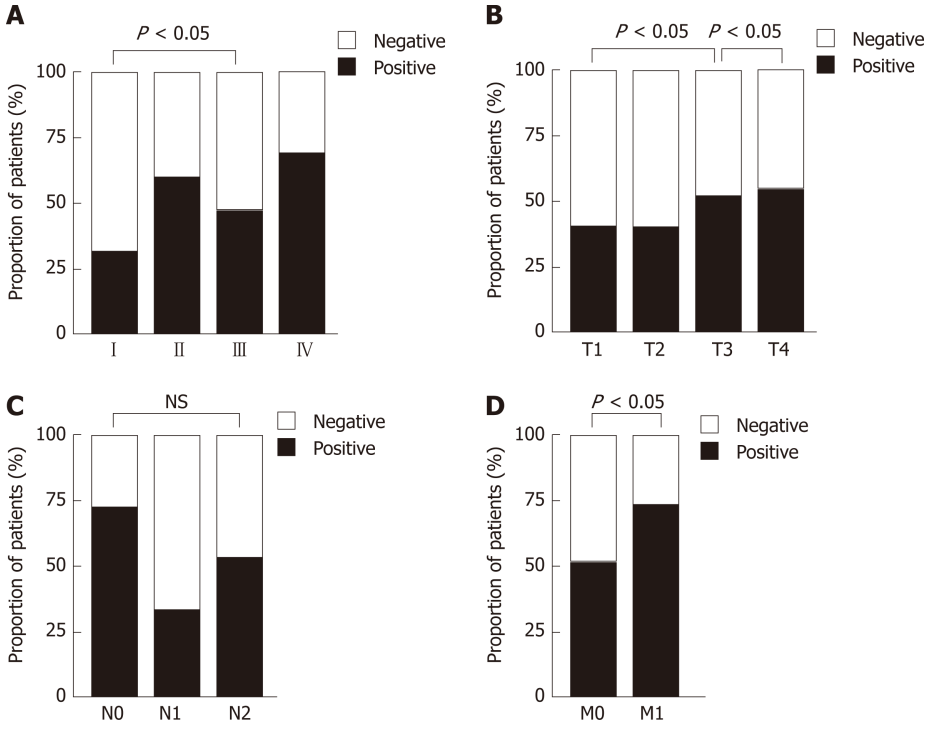


Figure 2 Graphical representations of the proportion of patients with positive and negative methylated septin 9 with different tumor status. A: Union for International Cancer Control stages; B: Primary tumor categories; C: Regional node categories; D: Distant metastasis categories. The statistical significance for difference of means is shown in *P* values and $\chi 2$ test).

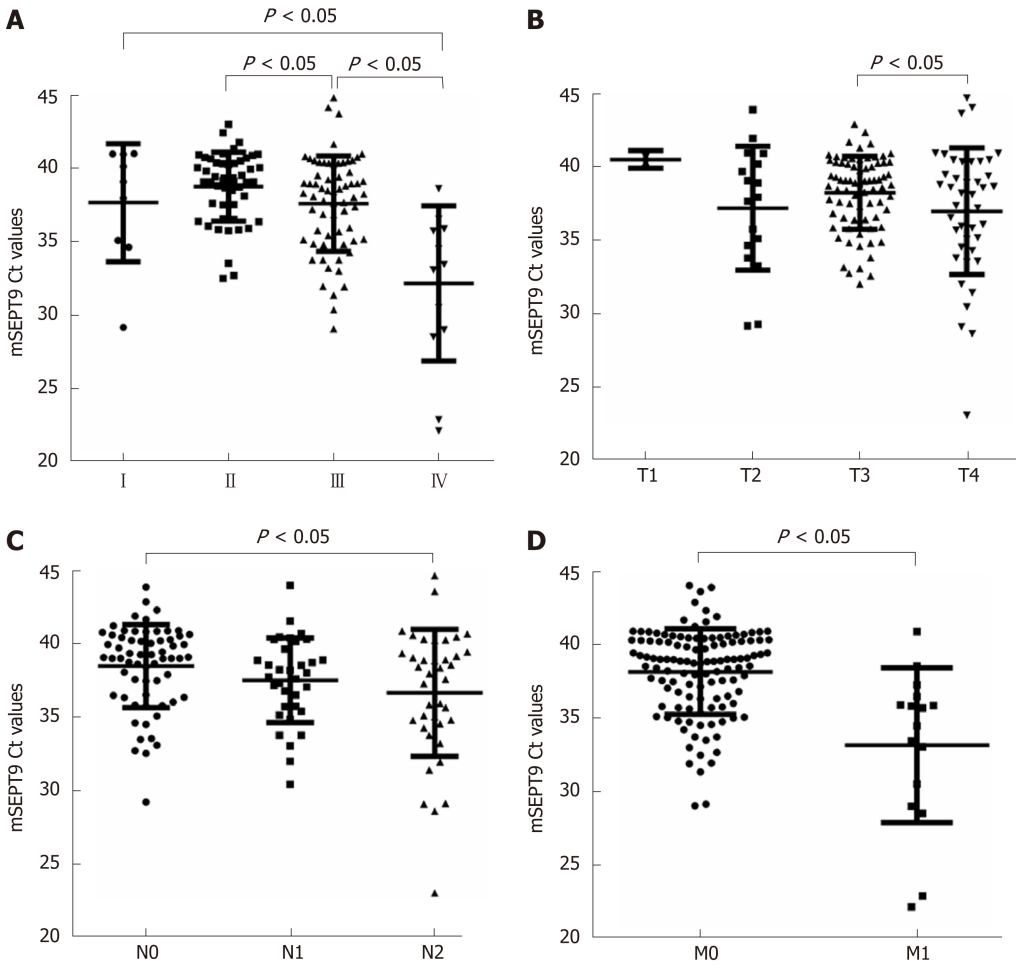
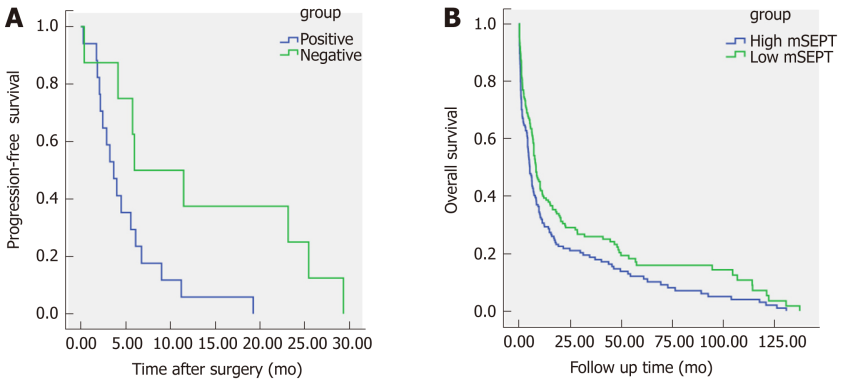


Figure 3 Graphical representations of methylated septin 9 Ct values in different tumor status. A: Union for International Cancer Control stages; B: Primary

tumor categories; C: Regional node categories; D: Distant metastasis categories. The statistical significance for difference of means is shown in *P* values and *t*-test. mSEPT9: Methylated septin 9.



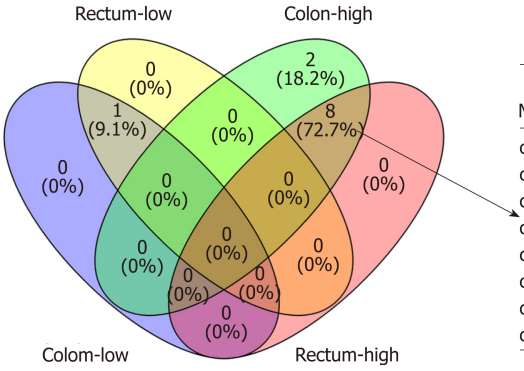
	Positive (n = 157)	Negative (n = 143)
Median survival	3.7	6.0
95%CI	2.14-5.19	0-13.87

P = 0.019

	Low mSEPT (n = 165)	High mSEPT (n = 165)
Median survival	8.1	5.1
95%CI	6.53-9.27	3.87-6.33

P = 0.008

Figure 4 Kaplan-Meier univariate survival curves according to methylated septin 9 status. A: Progression-free survival time; B: Overall survival. The statistical significance for difference of means shown in *P* values and Kaplan-Meier univariate analysis. mSEPT: Methylated septin; CI: Confidence interval.



Methylated sites	Colon mSEPT9 expression fold change/*P*	Rectum mSEPT9 expression fold change/*P*
cg02884239	$4.81/2.47e^{-16}$	5.63/0.0015
cg20275528	$5.31/3.33e^{-20}$	3.57/0.0012
cg19554255	$6.61/9.00e^{-19}$	6.92/0.0012
cg03804136	$11.65/1.15e^{-16}$	9.84/0.0137
cg17300544	$13.22/3.7e^{-17}$	13.27/0.0063
cg06848185	$17.11/1.61e^{-18}$	22.10/0.0015
cg16779463	$18.95/1.15e^{-16}$	16.74/0.0137
cg15044248	$25.14/1.45e^{-17}$	26.48/0.0063

Figure 5 Venn diagram of eight co-upregulated methylated septin 9 sites and one co-downregulated methylated septin 9 site in colon and rectum adenocarcinoma. "Rectum-low" and "Rectum-high" represented sites that showed low or high expression in rectum adenocarcinoma, and "Colon-low" and "Colon-high" represented sites that showed low or high expression in colon adenocarcinoma. Eight co-upregulated methylated septin 9 (mSEPT9) sites also showed mSEPT9 expression fold of rectum/ colon adenocarcinoma compared to normal subjects and corresponding *P* value. mSEPT: Methylated septin.

ARTICLE HIGHLIGHTS

Research background

The methylated septin 9 (mSEPT9) assay was the first blood-based test approved by the United States Food and Drug Administration as a colorectal screening test. Previous researchers found that mSEPT9 was a powerful screening, diagnostic, monitoring, and prognostic tool for German colorectal cancer (CRC) patients. However, the diagnostic and prognostic value of mSEPT9 in Chinese CRC patients is still unknown, and may be affected by differences in ethnicity and socioeconomic status.

Research motivation

To explore the diagnostic and prognostic value of serum mSEPT9 for Chinese CRC patients.

Research objectives

This study aimed to explore the diagnostic value of preoperative serum mSEPT9 in the Chinese population, and then assess the value of quantitative mSEPT9 levels for CRC staging. In addition, Chinese population and TCGA database information were combined to determine the prognostic significance of mSEPT9 by bioinformatics analyses.

Research methods
Three hundred fifty-four subjects (300 CRC, 54 normal) from China and 351 subjects (330 CRC, 21 normal) from the TCGA database including American Indian, Asian, Black, and African American populations were retrospectively analyzed. Preoperative mSEPT9 levels were quantified by quantitative methylation-specific polymerase chain reaction. Kaplan-Meier univariate assay was performed to analyze potential prognostic factors including overall survival (OS) and progression-free survival (PFS).

Research results
In Chinese CRC patients, positive mSEPT9 and quantitative mSEPT9 levels were strongly associated with clinico-pathological parameters. The patients with positive mSEPT9 showed a tendency toward lower PFS. Higher mSEPT9 levels were correlated with more distant metastasis among the TCGA database patients, and patients with high mSEPT9 levels showed a tendency toward lower OS.

Research conclusions
Testing for mSEPT9 is a powerful diagnostic and promising prognostic tool for Chinese CRC patients; it may add valuable information to current tumor staging and holds the potential to monitor CRC recurrence.

Research perspectives
This study assessed the correlation between clinicopathological characteristics and preoperative serum mSEPT9 in Chinese CRC patients and, further, to confirm the correlation between mSEPT9 levels and CRC prognosis by bioinformatics analyses. In addition, we analyzed methylated sites that were co-upregulated or co-downregulated in colon and rectum tumors, to provide the theoretical guidance for further research.

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World J Gastroenterol 2019 May 7; 25(17): 2110-2121

DOI: 10.3748/wjg.v25.i17.2110

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Clinical Trials Study

Beneficial effect of probiotics supplements in reflux esophagitis treated with esomeprazole: A randomized controlled trial

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Author contributions: Sun SD designed the research; Wang HY, Zhang X, and Zhang H performed the research; Sun SD and Wang HY contributed new reagents and analytic tools; Wang HY analyzed the data; Sun QH wrote the paper.

Supported by Shandong Provincial Medical and Health Technology Development Project, No. 2014ws0020.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of PKUCare Luzhong Hospital [2015-KY-003].

Clinical trial registration statement: This study is registered on the Chinese Clinical Trial Registry (No. ChiCTR1800018218).

Informed consent statement: All study participants, or their legal guardian, provided written informed consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that they have no competing interests.

Data sharing statement: No additional data.

CONSORT 2010 statement: CONSORT 2010 checklist of information to include when reporting a randomized trial has

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Abstract

BACKGROUND

Reflux esophagitis (RE) is a common digestive disorder, and its frequent recurrences cause significant physical pain and are financially burdensome to patients. However, studies on the natural history of treated RE are few. Although proton pump inhibitors (PPIs) as the first-line treatment provide notable symptomatic relief, disordered gut microbiota has been observed among PPI users. Probiotics are commonly administered to patients to regulate the disordered intestinal flora.

AIM

To evaluate the therapeutic effects in RE patients treated with a combination of esomeprazole and probiotics [*Bacillus subtilis* (*B. subtilis*) and *Enterococcus faecium* (*E. faecium*)].

METHODS

One hundred and thirty-four RE patients were randomized into two groups of 67 subjects each. The probiotics group was administered with esomeprazole 20 mg *b.i.d.* and live combined *B. subtilis* and *E. faecium* enteric-coated capsules 500 mg *t.i.d.* for eight weeks; the placebo group was administered with esomeprazole 20 mg *b.i.d.* and placebo for eight weeks. Subsequently, 12-wk follow-up was carried out on patients who achieved both endoscopic and clinical cure. Endoscopy, reflux diagnostic questionnaire (RDQ), gastrointestinal symptom rating scale (GSRS), and lactulose hydrogen breath test were performed to evaluate the therapeutic effects. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

been completed.

Manuscript source: Unsolicited manuscript

Received: January 11, 2019
Peer-review started: January 11, 2019
First decision: January 30, 2019
Revised: February 15, 2019
Accepted: February 22, 2019
Article in press: February 23, 2019
Published online: May 7, 2019

P-Reviewer: Kositamongkol P, Kressel A, Lee S
S-Editor: Ma RY
L-Editor: Wang TQ
E-Editor: Ma YJ

Sixty-six patients in the probiotics group and 64 patients in the placebo group completed the 8-wk treatment. The healing rate and RDQ score had no significant difference between the two groups ($P > 0.05$). However, the GSRS diarrhea syndrome score was decreased significantly in the probiotics group ($P = 0.002$), and the small intestinal bacterial overgrowth negative rate in the probiotics group was significantly higher than that in the placebo group ($P = 0.002$). Of 114 endoscopically and clinically cured patients, 96 completed the follow-up. The log-rank test showed that the time to relapse was shorter in the placebo group than in the probiotics group ($P = 0.041$). Furthermore, the therapy had a significant influence on relapse time, and the risk of relapse in the probiotics group was lower than that in the placebo group at any time point during the 12-wk follow-up (hazard ratio = 0.52, $P = 0.033$).

CONCLUSION
Esomeprazole combined with probiotics (*B. subtilis* and *E. faecium*) have a beneficial effect on RE treatment and patient management.

Key words: Proton pump inhibitors; Probiotics; Small intestinal bacterial overgrowth; Reflux esophagitis; Relapse

Core tip: Reflux esophagitis (RE) recurrences cause significant physical pain and financial burden to patients. Proton pump inhibitors (PPIs) are the first-line treatment for RE. Although PPIs provide notable symptomatic relief, their effects on the gut microbiota have drawn attention. In the present study, we evaluated the effectiveness of combining esomeprazole with probiotics [*Bacillus subtilis* (*B. subtilis*) and *Enterococcus faecium* (*E. faecium*)]. We found that the combined administration could reduce the incidence of small intestinal bacterial overgrowth and improve abdominal symptoms in patients with RE. It may also prolong the time to relapse, showing the potential of probiotics (*B. subtilis* and *E. faecium*) for the treatment and management of RE.

Citation: Sun QH, Wang HY, Sun SD, Zhang X, Zhang H. Beneficial effect of probiotics supplements in reflux esophagitis treated with esomeprazole: A randomized controlled trial. *World J Gastroenterol* 2019; 25(17): 2110-2121
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2110.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2110

INTRODUCTION

Reflux esophagitis (RE) is a common digestive disorder that occurs when gastric/duodenal contents flow pathologically into the esophagus, leading to inflammation, erosion, and ulceration of the esophageal mucosa. Frequent relapses are common with RE, resulting in significant physical pain and financial burden on patients. Studies on the treatment of RE are scarce[1,2]. The first-line treatment for RE is administration of proton pump inhibitors (PPIs)[3], which are the most commonly prescribed drugs worldwide. Some studies have reported complete responses in approximately 70%-80% of patients after eight weeks of PPI treatment[4].

Although PPIs provide notable symptomatic relief, their effects on the gut microbiota have gained recent attention. A large population-based cohort study showed a significant reduction in the abundance of gut flora and microbial diversity and an associated significant increase in the amount of oral and upper gastrointestinal (GI) tract bacteria among PPI users[5]. Profound changes have been observed in the gastric and intestinal microbiota of PPI users[6-9].

Small intestinal bacterial overgrowth (SIBO) refers to an elevated bacterial count that reflects changes in the composition and structure of the small intestine[5]. Many studies have reported an increased incidence of SIBO during PPI therapy[10]. SIBO presents with a variety of GI symptoms, such as diarrhea, abdominal distension, and constipation[11]. Many recent studies have shown that PPIs can cause symptoms of GI discomfort similar to those associated with SIBO[12-15].

Probiotics comprise microorganisms that enhance the integrity of the intestinal

mucosal barrier and balance the microbial ecosystem. This is achieved *via* probiotic competition with harmful bacteria and the production of metabolites that inhibit the growth of the harmful bacteria. Probiotics are commonly administered to patients with intestinal flora abnormalities.

This clinical trial aimed to evaluate the effectiveness of combining esomeprazole with probiotics [live combined *Bacillus subtilis* (*B. subtilis*) and *Enterococcus faecium* (*E. faecium*)] for the treatment of patients with RE by comparing the outcomes after eight weeks of treatment in a treatment group and a placebo group.

MATERIALS AND METHODS

Study subjects

From June 2015 to December 2017, 134 RE outpatients or gastroenterology inpatients in the PKUCare Luzhong Hospital were recruited in this trial. RE was diagnosed based on the 2013 Guidelines for the Diagnosis and Management of Gastroesophageal Reflux Disease[4]. The inclusion criteria were: (1) patients who consented to undergo esomeprazole treatment, were not previously on PPI, or have stopped PPI treatment for at least 6 mo, and were aged 18-65 years; (2) patients who have not taken antibiotics, probiotics, lactulose, other antacids, or drugs that increase GI motility nor undergone an enema in the past 4 wk; (3) normal hepatic and renal function; and (4) SIBO negative on the lactulose hydrogen breath test (LHBT). The exclusion criteria were: (1) history of cirrhosis, renal impairment, tumors, thyroid disease, diabetes, Crohn's disease, or ulcerative colitis; (2) comorbid hiatal hernia, peptic ulcer disease, esophageal stricture, diarrhea, malabsorption, and constipation due to liver, gallbladder, and pancreatic diseases; (3) history of GI or abdominal surgery; (4) pregnant or lactating women; (5) patients undergoing treatment with immune suppressants; and (6) patients who fulfilled the diagnosis of irritable bowel disease (IBS) according to the Rome III criteria, or patients who did not meet the diagnostic criteria but had persistent abdominal distension, diarrhea, or constipation for ≥ 3 mo. The enrollment flowchart is displayed in Figure 1.

Ethics

All subjects signed an informed consent form. This study was reviewed and approved by the ethics committee of PKUCare Luzhong Hospital (2015-KY-003) and registered on the Chinese Clinical Trial Registry (No. ChiCTR1800018218).

Endoscopy

Endoscopic findings were classified according to the Los Angeles Classification grading system (grade A: ≥1 mucosal break < 5 mm; grade B: ≥ 1 mucosal break > 5 mm; grade C: mucosal breaks extending between the tops of two mucosal folds, but < 75% of the circumference; grade D: mucosal breaks extending for > 75% of the circumference). Improvement in the endoscopic findings to grade N (normal) is defined as healing.

LHBT

The EC60 Gastrolyzer 2 (United Kingdom) was used for the test. The subject first exhaled once to measure the baseline value before taking 200 mL of lukewarm water and 10 mL of lactulose (lactulose oral solution, Laiyang Jiangbo Pharmaceutical Co., Ltd., 10 mL/vial). After gargling, the patient exhaled once every 20 min for 3 h. A normal LHBT value was defined as baseline value < 20 ppm and a maximum peak value of < 20 ppm greater than the baseline value. A positive result was defined as classical double peak and (or) a fusion peak waveform.

Reflux diagnostic questionnaire (RDQ)

The RDQ was used to assess the subjective reflux symptoms covering a 1-wk recall period. RDQ is categorized into four symptom clusters depicting heartburn, chest pain, acid reflux, and food reflux. The total RDQ scores (eight items) were calculated. Patients with RDQ ≥ 12 points were considered to have a relapse[16].

GI symptom rating scale (GSRS)

The GSRS is a disease-specific instrument, containing 15 items, each rated on a seven-point Likert scale from which one represents no discomfort and seven represents very severe discomfort[17]. The 15 GSRS items break down into the following five symptom clusters: abdominal pain (abdominal pain, hunger pain, and nausea); reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools, and urgent need for defecation), indigestion syndrome (borborygmus, abdominal distension, eructation, and increased flatus), and constipation syndrome (constipation,

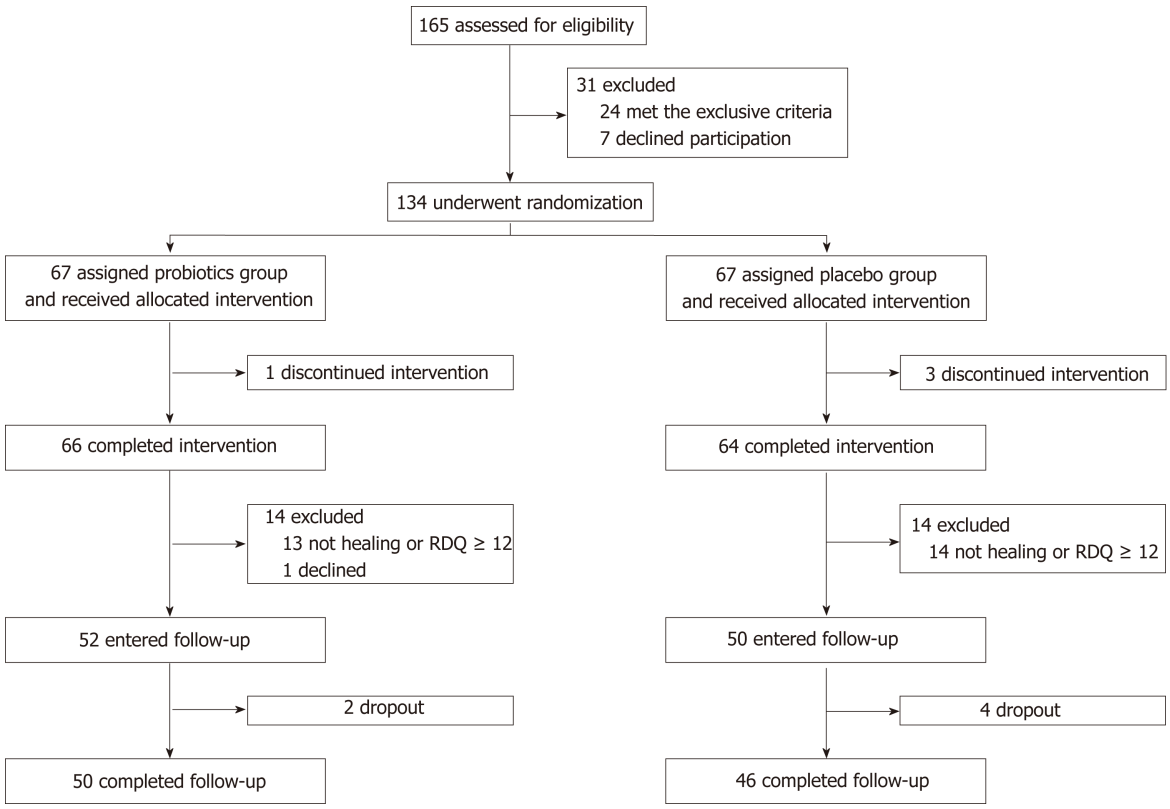

Figure 1 Trial profile. The probiotics group refers to esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* treatment; the placebo group refers to esomeprazole 20 mg *b.i.d.* and placebo treatment. RDQ: Reflux diagnostic questionnaire.

hard stools, and feeling of incomplete evacuation).

Clinical evaluation and intervention

Phase 1: A random number table was used to divide the 134 RE patients into two groups of 67 subjects each. Esomeprazole is the first choice of PPI, having strong and lasting acid suppression effect. Medilac-s are live combined *B. subtilis* and *E. faecium* enteric-coated capsules. These two kinds of bacteria are regular members of the intestinal flora of healthy people. Taking this product can directly supplement normal physiological living bacteria, inhibit the excessive reproduction of harmful bacteria in the intestinal tract, and adjust the intestinal flora, which is applied widely in the clinic. The dosage of the medicine was determined by the published drug instructions[18]. The placebo was provided by the Pharmacy Department from the PKUCare Luzhong Hospital. The dosage form, appearance, size, and color of the placebo were completely identical with the drug. The drugs conform to China's Good Manufacture Practice of Medical Products[19]. Patients in the placebo group took 20 mg of esomeprazole (Nexium, AstraZeneca PLC) orally twice a day and placebo (white starch capsules) thrice a day for eight weeks. Patients in the probiotics group took 20 mg of esomeprazole orally twice a day and 500 mg of live combined *B. subtilis* and *E. faecium* enteric-coated capsules (Hanmi Pharmaceutical Co., Ltd) thrice a day for 8 wk. The treatment was single blinded. Patients did not know their assigned groups. Observation for medication compliance (PPI and probiotic/placebo) was performed twice a week through phone, by asking the parents about compliance. Poor compliance was defined as missed doses for ≥ 3 d.

Phase 2: Patients who achieved endoscopic and clinical cure (RDQ < 12) during phase 1 entered the follow-up. The follow-up endpoint was defined as symptomatic recurrence (RDQ ≥ 12) or the end of the 12-wk follow-up (week 20).

Endoscopic evaluation was performed at baseline and repeated at the end of the treatment (week 8) to verify healing. GSRS was completed at baseline and week 8. RDQ and LHBT were completed at baseline before treatment, week 8, and the follow-up endpoint. The same physician performed an initial clinical evaluation and the following medical appointments. All subjects received telephone or outpatient follow-up once every two weeks. We assessed the therapeutic effect of treatments using the change in endoscopic evaluation and RDQ at the end of therapy and the end of follow-up (primary outcomes). Changes in GSRS and LHBT results were considered

the secondary outcomes.

Adverse events and disallowed medication

Adverse events were monitored throughout the study. Patients were not allowed to consume any other probiotics or prebiotics, and they were instructed to continue their usual eating and living habits. The use of antacids or motility-increasing drugs was stopped during the follow-up period unless the symptom relapsed. Concomitant use of medications was allowed, providing their registered medication intake.

Statistical analysis

All data were processed and analyzed with the R Studio (version 3.4.3, R Studio Inc., Boston, United States), and the packages 'survival' (version 2.42-6), 'survminer' (version 0.4.3), and 'dplyr' (version 0.7.7) were used to run and visualize statistical tests. Statistical significance was defined as $P < 0.05$. Quantitative data that conformed to a normal distribution are expressed as the mean ± standard deviation, and *t*-test was used for intergroup comparison. Chi-squared test was applied to frequency data for intergroup comparison. Kaplan-Meier analysis was utilized to analyze the cumulative relapse rate of RE. Cox regression analysis was conducted considering the prognostic variables of clinical characteristics at entry and initial treatment therapy to explore the effect of other factors on the relative risk of relapse.

The statistical power calculation was carried out to estimate the sample size for the superiority trial. According to our review of studies, relapse rates of patients with healed lesions have been reported to be 54% to 66.2% at 12 wk after drug therapy was withdrawn[2,20,21], so our estimation of the average relapse rate for the placebo group was 60%. Also, cured RE patients who received an additional maintenance treatment had a relapse rate of 10% at 12 wk and 28.4% to 30% at 32 wk after drug therapy was stopped[1,22]. Given that the therapeutic effect of probiotics supplements on RE recurrence had never been studied and the probiotics are not antacid, we took 30% as our estimation of the relapse rate for the probiotics group. Hence, we estimated that the average relapse rate was 30%. With a two-tailed test of $\alpha = 0.05$ and $1 - \beta = 0.80$, the calculation indicated that a sample size of 40 for each group would be sufficient. To power our trial to be able to detect the difference between groups maximumly, we included as many patients as possible within our study budget rather than just meeting the minimum sample size requirement of 40 patients[23].

RESULTS

Phase 1: Placebo-controlled study

Clinical features at baseline: One and three patients discontinued the intervention in the probiotics and placebo groups, respectively. Finally, 130 patients completed the study, of which 66 and 64 patients were in the probiotics and placebo groups, respectively (Figure 1). Baseline characteristics and questionnaire scores are shown in Table 1. There were no statistically significant differences in age, sex, body mass index, smoking history, waist circumference, esophagitis grade, and GSRS and RDQ scores between the two groups at baseline ($P > 0.05$ for all). The general status of patients in both groups was balanced, and the experiment results were comparable.

Intervention: Figure 2 shows the RDQ scores, GSRS scores, and endoscopic healing rates in the probiotics and placebo groups after eight weeks of treatment. In the probiotics group, total RDQ score was 9.29 ± 6.65, total GSRS score was 31.59 ± 8.95, GSRS abdominal pain score was 5.45 ± 3.39, GSRS reflux syndrome score was 4.71 ± 3.20, GSRS diarrhea syndrome score was 6.20 ± 3.88, GSRS indigestion syndrome score was 8.58 ± 4.57, and GSRS constipation syndrome score was 5.05 ± 1.83. In the placebo group, they were 9.86 ± 6.84, 32.94 ± 6.04, 5.11 ± 2.57, 5.16 ± 2.72, 7.94 ± 2.36, 9.82 ± 5.04, and 5.02 ± 2.72, respectively. There was no significant difference between the two groups in RDQ score ($P = 0.631$), total GSRS score ($P = 0.317$), GSRS abdominal pain score ($P = 0.521$), GSRS reflux syndrome score ($P = 0.390$), GSRS indigestion syndrome score ($P = 0.144$), and GSRS constipation syndrome score ($P = 0.941$). However, the GSRS diarrhea syndrome score was decreased significantly in the probiotics group ($P = 0.002$).

Endoscopic examinations were performed after 8-wk treatment. The endoscopic healing rates in the probiotics group at week 8 were 100% (26/26), 95.5% (21/22), 69.2% (9/13), and 40.0% (2/5) in patients with grades A, B, C, and D, respectively; in the placebo group, the healing rates were 100% (29/29), 95.2% (20/21), 54.5% (6/11), and 33.3% (1/3) in patients with grades A, B, C, and D, respectively. There was no significant difference in the healing rate between the probiotics and placebo groups in all grades (grade A: $P > 0.05$, grade B: $P = 0.974$; grade C: $P = 0.495$; grade D: $P =$

Table 1 Clinical characteristics of patients in the probiotics and placebo groups at baseline

Characteristic		Probiotics group (*n* = 66)	Placebo group (*n* = 64)	*P*-value
Age (yr)		41.76 ± 9.38	41.89 ± 9.75	0.937
Male *n* (%)		39 (59.1)	40 (62.5)	0.691
BMI (kg/m^2)		24.61 ± 3.51	23.90 ± 3.14	0.230
Smoking *n* (%)		12 (18.2)	10 (15.6)	0.698
Waist circumference (cm)		78.68 ± 5.03	78.84 ± 6.49	0.874
RDQ score		19.41 ± 4.23	18.44 ± 5.17	0.244
GSRS score	Abdominal pain	6.38 ± 2.64	6.48 ± 3.20	0.846
	Reflux	10.35 ± 2.48	10.31 ± 2.68	0.937
	Diarrhea	6.44 ± 1.97	6.89 ± 2.39	0.242
	Indigestion	7.53 ± 2.67	7.03 ± 2.17	0.245
	Constipation	5.48 ± 1.28	5.34 ± 2.13	0.647
Esophagitis grade at baseline (*n*)	A	26	29	0.495
	B	22	21	0.950
	C	13	11	0.712
	D	5	3	0.493

BMI: Body mass index; RDQ: Reflux diagnostic questionnaire; GSRS: Gastrointestinal symptom rating scale.

0.849).

Phase 2: Relapse after stopping treatment

Of 114 eligible healed patients, 102 entered phase 2 (1 refused, 11 with RDQ ≥ 12), 96 completed the follow-up, 50 were from the probiotics groups, and 46 were from the placebo group. At the endpoint of the follow-up, 22 patients had a relapse in the probiotics group, whereas 28 patients had a relapse in the placebo group. Figure 3 shows the cumulative rate of symptomatic recurrence. The result of the log-rank test showed that the two curves differed significantly (P = 0.041), which means that the treatment therapy has a significant influence on relapse time, and the time to relapse is shorter in the placebo group than in the probiotics group. Among the recurrent patients, RDQ scores in the placebo group (17.11 ± 2.85) was higher than that in the probiotics group (15.40 ± 2.34). There was a significant difference in outcome between the two groups (P = 0.024).

Cox regression analysis on the relapse data showed that the treatment therapy and esophagitis grade at entry had a significant effect on the recurrence. The risk of relapse in the probiotics group was lower than that in the placebo group at any time point during the 12-wk follow-up [hazard ratio (HR) = 0.52, P = 0.033]. Patients with esophagitis grade D had a higher risk of relapse than patients with esophagitis grade A at entry (HR = 79.85, P < 0.001). No other evidence was observed that gender, smoking, baseline RDQ score, or waistline would influence the rate of relapse significantly (Figure 4).

SIBO in RE patients

All the patients underwent LBHT testing at baseline, week 8, and the follow-up endpoint. At baseline, all the patients were SIBO negative. After the 8-wk treatment, the SIBO negative rate in the probiotics group (84.8%, 56/66) was higher than that in the placebo group (60.9%, 39/64); the difference between the two groups was statistically significant (P = 0.002). At the endpoint of follow-up, the SIBO negative rate was slightly increased in both groups, 88.0% (44/50) in the probiotics group and 65.2% (30/46) in the placebo group. The percentage of SIBO negative patients in both groups did not change significantly with time (Figure 5). The rate of relapse in SIBO positive patients (45.9%, 34/74) was higher than that in SIBO negative patients (72.7%, 16/22) at the endpoint of follow-up (P = 0.027).

Adverse events and withdrawals

Four patients suffered adverse events in phase 1 and discontinued the intervention. One in the probiotics group and two in the placebo group had nausea and vomiting. One in the placebo group had dermatitis. Minor adverse events were recorded and evaluated by GSRS. In the follow-up period, two patients in the probiotic group and two in the placebo group withdrew for taking drugs that may influence the gut microbiota (antibiotics and probiotics). Two in the placebo group were lost to follow-

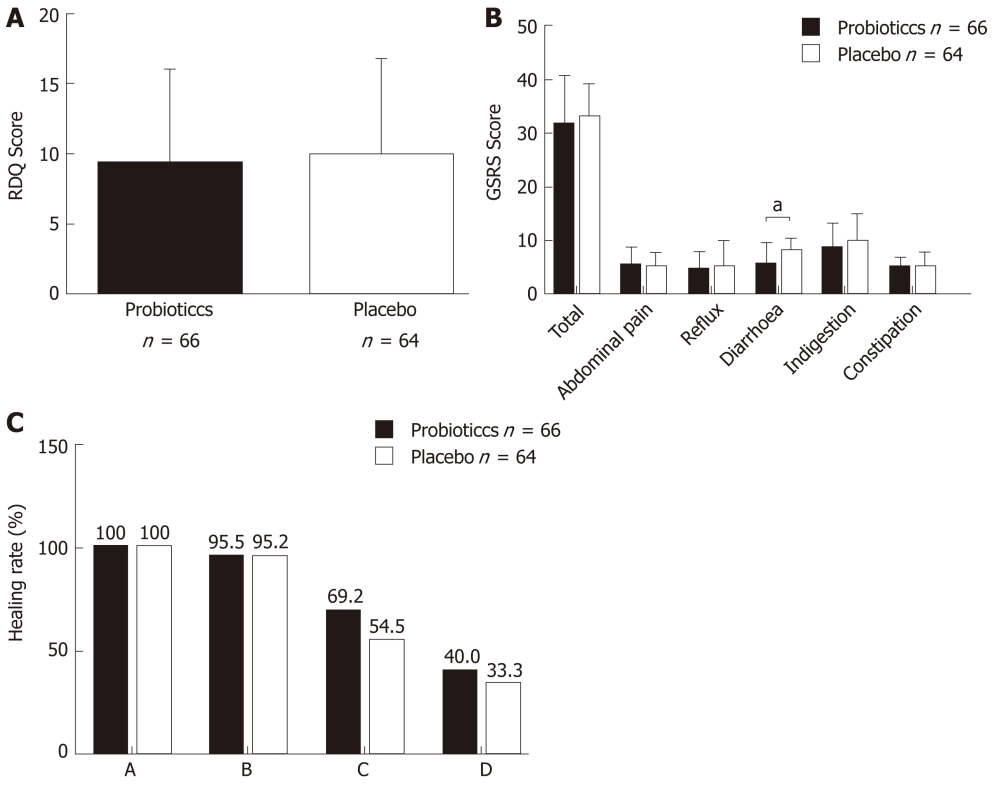

Figure 2 Efficacy of esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* A: Reflux diagnostic questionnaire scores, B: Gastrointestinal symptom rating scale scores, C: Endoscopic healing rates in the probiotics and placebo groups after eight weeks of treatment. Probiotics refers to esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* treatment; placebo refers to esomeprazole 20 mg *b.i.d.* and placebo treatment. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. RDQ: Reflux diagnostic questionnaire; GSRS: Gastrointestinal symptom rating scale.

up.

DISCUSSION

To our knowledge, this is the first randomized controlled clinical trial to evaluate the impact of disordered gut microbiota on RE, as well as the therapeutic effects of probiotic supplements in patients with RE.

In our study, 8-wk treatment with esomeprazole (20 mg *b.i.d.*) and Medilac-s, live combined *B. subtilis* and *E. faecium* enteric-coated capsules (500 mg *t.i.d.*), reduced the incidence of SIBO and improved the diarrhea syndrome in RE patients. The endoscopic healing rates were higher in cases with low-grade esophagitis but lower in cases with more severe baseline esophagitis. The healing rates of RE patients in the probiotics and placebo groups were similar. The probiotics supplements may not influence the acid-suppression efficacy because esomeprazole is the most effective and long-lasting antacid PPI[24].

Acid suppression with PPIs has been suggested to be a precursor to the development of SIBO. In a clinical study on patients with functional dyspepsia, Tsuda *et al*[25] found that 4 wk of PPI use caused SIBO. Oana *et al*[26] conducted a clinical trial on pediatric gastroesophageal reflux disease (GERD) patients administered probiotics and PPI for 12 wk and found that probiotics administration decreased the rate of dysbiosis in children treated with PPI. Jacobs C *et al*[27] conducted a study focusing on the risk factors of SIBO. Studies showed that PPI use was an independent risk factor for SIBO. However, some other clinical trials showed different conclusions. In one prospective study, quantitative cultures of duodenal aspirates were performed to detect SIBO. Giamarellos-Bourboulis *et al*[28] found that PPI intake could not increase SIBO. A double-blind placebo-controlled randomized trial of the effect of probiotics on SIBO in children treated with omeprazole conducted by Badriul Hegar *et al*[24] found that probiotics did not decrease the risk of developing SIBO. However, it is notable that in this trial the subjects were children and they took PPIs for 4 wk. The dosage and duration of therapy in this study were lower and shorter than those in reports on adults[29,30]. The duration of PPI therapy was directly related to SIBO

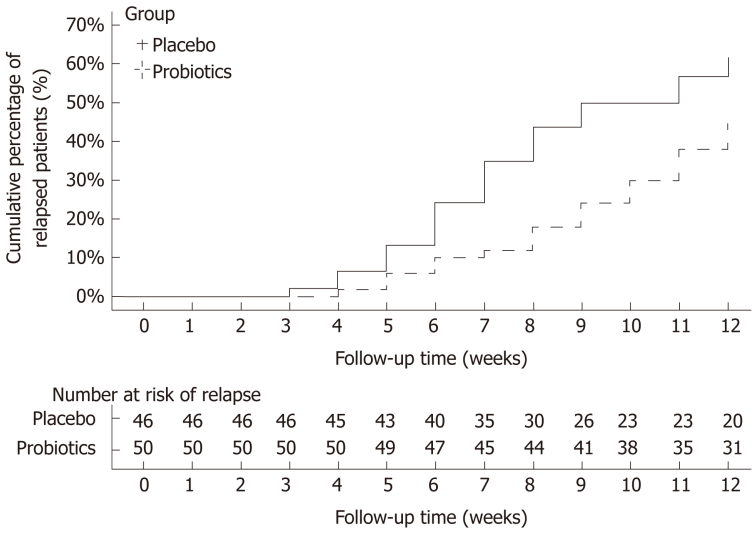

Figure 3 Cumulative event curves of the recurrence of reflux esophagitis in the probiotics and placebo groups. Probiotics refers to esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* treatment; placebo refers to esomeprazole 20 mg *b.i.d.* and placebo treatment.

incidence[31]. Moreover, two meta-analyses reported that the use of PPI could increase the risk of SIBO[32,33].

Del Piano *et al*[34] found *Escherichia coli* (*E. coli*) in the gastric juice of patients who used PPI for more than 3 mo, and given that *E. coli* is extremely rare in the stomach of healthy people, this result indicated that reducing gastric juice pH would result in excessive growth of stomach-associated bacteria (such as *E. coli*) and increase the risk of infection and intestinal diseases. A recent study demonstrated that excessive bacterial growth might be due to reduced intragastric bacterial obliteration[35]. A cohort study by Ardatskaia *et al*[36] found no differences in the incidence of SIBO between patients with atrophic gastritis and patients with GERD following long-term PPI treatment; however, the rates in both groups were higher than in healthy populations, which also proved that a deficiency in gastric acid can result in reduced complexity of gut microbial communities. Long-term PPI use had been shown to decrease *Bacteroides* and increase *Firmicutes* in the gut, which may predispose an individual to the development of *Clostridium difficile* infection (CDI)[37]. A crossover trial conducted by Daniel *et al*[38] showed that significant changes during PPI use in taxa associated with CDI (increased *Enterococcaceae* and *Streptococcaceae*, and decreased *Clostridiales*) and taxa associated with GI bacterial overgrowth (increased *Micrococcaceae* and *Staphylococcaceae*) provided a mechanism by which PPIs predispose an individual to CDI. A study involving multiple methods of microbiota analysis, including quantitative RT-PCR, 16S rRNA sequencing analysis, and a metagenomic analysis, showed that bacteria such as *Streptococcus*, which are present in the human oral cavity, throat, and nasal cavity, increased in the intestine, implying that bacterial translocation, as well as enteric infections, may have occurred. This may be because PPIs reduced stomach acidity, and the barrier function is weakened[9]. The use of PPIs favors a relative excess of *Streptococcus* and *Campylobacteriosis*, and this might explain the persistence of dyspeptic and diarrhea symptoms in patients on PPI therapy[7,39,40].

On the other hand, a 2-wk course of Lactobacillus supplements in patients on long-term PPI treatment (>12 mo) has been shown to significantly reduce total bacterial count, proving the beneficial effects of probiotics in clinical treatment[34]. Del Piano *et al* believed that *Lactobacillus* and lactic acid bacteria had inhibitory effects on *Coliforms*. When patients on long-term PPI treatment were supplemented with probiotics, their *Enterococcus faecalis*, *E. coli*, mold, and yeast counts were all drastically reduced[31]. These findings proved that probiotics could regulate gut microbiota.

In our research, the addition of a probiotic combination (*B. subtilis* and *E. faecium*) to esomeprazole therapy led to a decrease in SIBO compared to that with the placebo, and the abdominal symptoms were also alleviated. This probiotic, Medilac-s, contains two live probiotics, combined *B. subtilis* and *E. faecium*, which can be stored at room temperature. They are constituents of normal intestinal flora in healthy people. They directly supplement normal intestinal flora, inhibit excessive proliferation of harmful bacteria in the gut, and regulate gut microbiota. We found that treatment with combined esomeprazole and live combined *B. subtilis* and *E. faecium* enteric-coated capsules had prophylactic effects on SIBO.

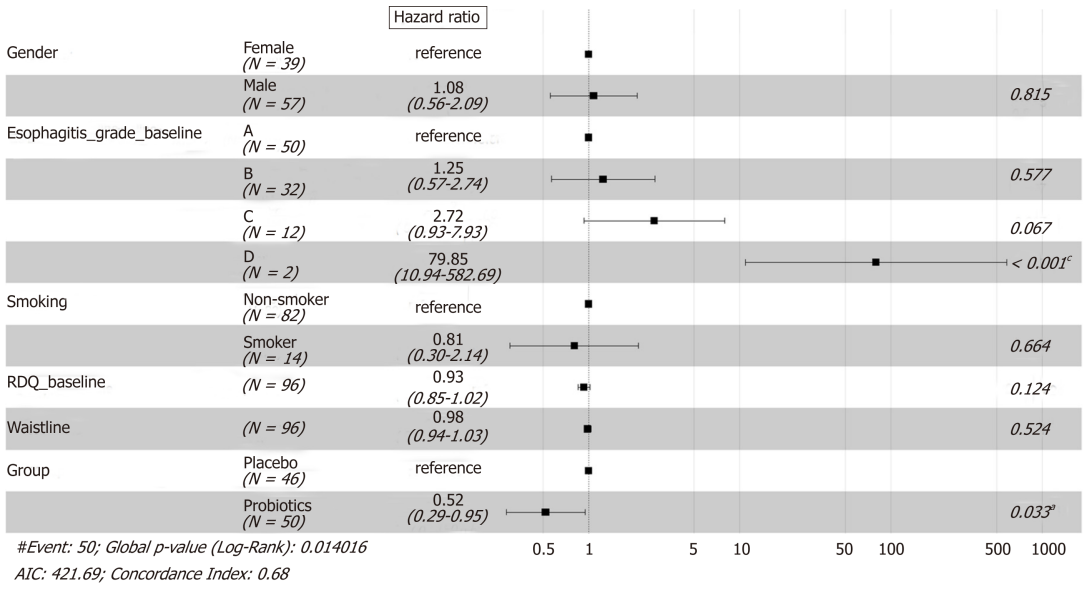

Figure 4 Forest plot for Cox proportional hazards model applied to the followed patients. Probiotics refers to esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* treatment; placebo refers to esomeprazole 20 mg *b.i.d.* and placebo treatment. [a]*P* < 0.05; [b]*P* < 0.01; [c]*P* < 0.001. RDQ: Reflux disease questionnaire; AIC: Akaike information criterion.

Although this combination of drugs did not increase the healing rate of esophagitis, the time to relapse was prolonged for 12 wk after PPI therapy withdrawal. Moreover, in the follow-up research, patients with SIBO had higher risks of symptomatic relapse than SIBO-negative patients. Cox regression analysis showed that the therapy administered (placebo or not) and esophagitis grade D were significant risk factors for recurrence of reflux symptoms. The possible explanation for this may be that a higher reflux recurrence rate is the result of changes in GI motility caused by SIBO. Akiho *et al*[41]carried out a study on IBS and found that Th2 cytokines could induce smooth muscle hypercontractility during intestinal infection. Th2 cytokines also induced transforming growth factor (TGF)-β1 expression and elevations in cyclooxygenase-2 and prostaglandin E2 levels in smooth muscle cells, resulting in intestinal motility disorder. German *et al*[42] employed a dog SIBO model and found that TGF-β1 and tumor necrosis factor (TNF)-α mRNA expression levels were decreased after SIBO treatment with antibiotics, *i.e.*, SIBO resulted in enhanced duodenal mucosal immune responses in dogs. SIBO-induced mild chronic inflammatory reactions and immune responses persistently acted persistently on smooth muscles in the GI tract, resulting in functional impairment, which simultaneously caused GERD or IBS-like symptoms. A study by Tugtepe *et al*[43] found impaired smooth muscle activity in the esophagus in a rat model of chronic RE. Currently, peristaltic abnormalities are present in 40%-50% of GERD patients[44]. Changes in gut microbiota may result in varying effects on gut mucosa and activate the immune and inflammatory response systems in the GI tract, resulting in functional impairment in the digestive and nervous systems, as well as visceral hypersensitivity, and impaired GI peristalsis. The above studies may partially explain why SIBO is associated with a higher recurrence rate of reflux symptoms and how a probiotics supplement can reduce the risks of relapse up to 12 wk after PPI withdrawal. In the future, further studies are needed to examine the pathophysiological mechanisms. Our study provides corroborated clinical trial materials as a basis for these studies.

Furthermore, a correlation between the severity of esophageal erosions and symptom relapse has been demonstrated in our study. Patients with SIBO are more likely to relapse. However, there were only two patients who were followed, and both of them relapsed, resulting in a wide confidence interval. More patients with esophagitis grade D are needed to verify this conclusion.

The significant strength of the present study was the strict exclusion criteria, wherein patients with hiatal hernia, GERD-predisposition, or bowel disorder were not recruited in order to ensure a homogeneous study group. A limitation of this study was the fact that we did not use jejunal cultures for SIBO assessment. Culture of the jejunal aspirate is recognized as the most direct method for diagnosing SIBO[45]. However, obtaining and culturing of jejunal aspirates are time-consuming and costly. In patients with isolated distal SIBO, SIBO could remain undiagnosed despite using jejunal cultures. Because of all of these disadvantages, LHBT was used in this study as

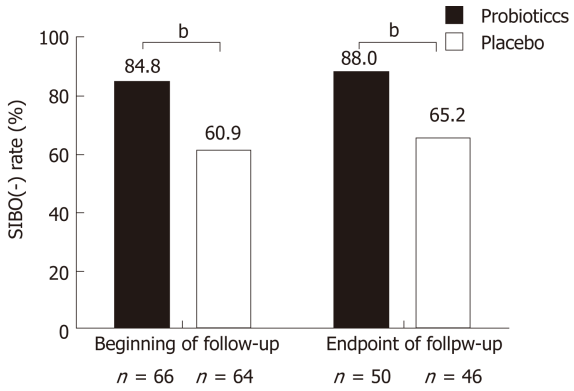

Figure 5 Proportion of patients without small intestinal bacterial overgrowth at the beginning and endpoint of follow-up. Probiotics refers to esomeprazole 20 mg *b.i.d.* and live combined *Bacillus subtilis* and *Enterococcus faecium* enteric-coated capsules 500 mg *t.i.d.* treatment; placebo refers to esomeprazole 20 mg *b.i.d.* and placebo treatment. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{c}P < 0.001$. SIBO: Small intestinal bacterial overgrowth.

an indirect but reliable alternative test to assess SIBO. Another limitation is that this was a single-center study with a limited sample size. Furthermore, the dietary habits of the included patients may affect the morbidity of RE and SIBO, and the effects of only *B. subtilis* and *E. faecium* probiotics on gut microbiota were studied. Furthermore, we did not perform endoscopy on asymptomatic patients after primary healing was achieved, and as a result, we were not able to detect asymptomatic relapses of esophagitis erosions. Therefore, the actual rate of mucosal relapse could not be determined in our study.

The combined administration of probiotics (*B. subtilis* and *E. faecium*) and esomeprazole could reduce the incidence of SIBO and improve abdominal symptoms in patients with RE. It may also prolong the time to relapse, showing the potential of probiotics (*B. subtilis* and *E. faecium*) for the treatment and management of RE.

ARTICLE HIGHLIGHTS

Research background

Profound changes have been observed in the gastric and intestinal microbiota of proton pump inhibitor users. Probiotics are commonly administered to patients with intestinal flora abnormalities. No prior studies have been conducted to evaluate the therapeutic effects of probiotics [*Bacillus subtilis* (*B. subtilis*) and *Enterococcus faecium* (*E. faecium*)] on patients with reflux esophagitis (RE).

Research motivation

We conducted a randomized controlled clinical trial to evaluate the impact of disordered gut microbiota on RE as well as the therapeutic effect of probiotics supplements on patients with RE.

Research objectives

This clinical trial aimed to study the RE patients treated with the combination of probiotic (*B. subtilis* and *E. faecium*) and esomeprazole.

Research methods

This study included 134 patients with RE who met the criteria. In phase 1, patients were divided into two groups. The probiotics group was given esomeprazole and live combined *B. subtilis* and *E. faecium* enteric-coated capsules for eight weeks, and the placebo group was given esomeprazole and placebo for eight weeks. Endoscopic evaluation, gastrointestinal symptom rating scale (GSRS), reflux diagnostic questionnaire (RDQ), and lactulose hydrogen breath test (LHBT) were performed at the end of the treatment. In phase 2, patients who achieved endoscopic and clinical cure (RDQ < 12) entered the follow-up. RDQ and LHBT were completed at the follow-up endpoint.

Research results

After eight-week treatment, the GSRS diarrhea syndrome score was decreased significantly in the probiotics group, and the small intestinal bacterial overgrowth (SIBO) negative rate in the probiotics group was significantly higher than that in the placebo group. Furthermore, the therapy had a significant influence on relapse time, and the risk of relapse in the probiotics group was lower than that in the placebo group at any time point during the 12-wk follow-up (hazard ratio = 0.52). However, only *B. subtilis* and *E. faecium* as probiotics were studied on gut microbiota in our study. More kinds of probiotics should be studied.

Research conclusions

The combined administration of probiotics (*B. subtilis* and *E. faecium*) and esomeprazole could reduce the incidence of SIBO and improve abdominal symptoms in patients with RE. It may also prolong the time to relapse, showing the potential of probiotics (*B. subtilis* and *E. faecium*) for the treatment and management of RE.

Research perspectives

The limitation of this study is the fact that we did not use jejunal cultures for SIBO assessment and did not perform endoscopy on asymptomatic patients after primary healing was achieved. Additional randomized controlled trials are needed to study more probiotics and different dosages, and prolong the follow-up time to evaluate the long-term effect.

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World Journal of Gastroenterology

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World J Gastroenterol 2019 May 7; 25(17): 2122-2132

DOI: 10.3748/wjg.v25.i17.2122

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Transitions of care across hospital settings in patients with inflammatory bowel disease

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Author contributions: Warren LR, Clarke JM, Arora S, Arebi N and Darzi A contributed to study conception and design; Warren LR, Clarke JM, contributed to data acquisition and writing of article; Warren LR, Clarke JM, and Barahona M contributed to data analysis and interpretation; Barahona M contributed to editing of article; Arora S, Arebi N, Darzi A and Barahona M contributed to review and final approval of article.

Supported by grants fromthe National Institute for Health Research (NIHR) Imperial Patient Safety and Translational Research Centre (PSTRC) and the Peter Sowerby Foundation. Infrastructure support for this research was provided by the NHIR Imperial Biomedical Research Centre (BRC). MB acknowledges support from EPSRC [grant number EP/N014529/1] supporting the EPSRC Centre for Mathematics of Precision Healthcare. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health.

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Abstract

BACKGROUND

Inflammatory bowel disease (IBD) is a chronic, inflammatory disorder characterised by both intestinal and extra-intestinal pathology. Patients may receive both emergency and elective care from several providers, often in different hospital settings. Poorly managed transitions of care between providers can lead to inefficiencies in care and patient safety issues. To ensure that the sharing of patient information between providers is appropriate, timely, accurate and secure, effective data-sharing infrastructure needs to be developed. To optimise inter-hospital data-sharing for IBD patients, we need to better understand patterns of hospital encounters in this group.

AIM

To determine the type and location of hospital services accessed by IBD patients in England.

METHODS

statement: This study received local ethical approval through the Imperial College Research Ethics Committee [17IC4178].

Informed consent statement: This study used administrative data that was not identifiable. Informed consent was not applicable.

Conflict-of-interest statement: There are no financial conflicts of interest declared by the authors.

Data sharing statement: HES data are available on application to the NHS Digital (https://digital.nhs.uk).

STROBE statement: This study followed the guidelines of the STROBE statement.

Manuscript source: Unsolicited manuscript

Received: January 17, 2019
Peer-review started: January 18, 2019
First decision: January 30, 2019
Revised: February 5, 2019
Accepted: February 22, 2019
Article in press: February 23, 2019
Published online: May 7, 2019

P-Reviewer: Ierardi E, Serban ED, Sipos F, Suzuki H, Tsujikawa T
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

This was a retrospective observational study using Hospital Episode Statistics, a large administrative patient data set from the National Health Service in England. Adult patients with a diagnosis of IBD following admission to hospital were followed over a 2-year period to determine the proportion of care accessed at the same hospital providing their outpatient IBD care, defined as their 'home provider'. Secondary outcome measures included the geographic distribution of patient-sharing, regional and age-related differences in accessing services, and type and frequency of outpatient encounters.

RESULTS

95055 patients accessed hospital services on 1760156 occasions over a 2-year follow-up period. The proportion of these encounters with their identified IBD 'home provider' was 73.3%, 87.8% and 83.1% for accident and emergency, inpatient and outpatient encounters respectively. Patients living in metropolitan centres and younger patients were less likely to attend their 'home provider' for hospital services. The most commonly attended specialty services were gastroenterology, general surgery and ophthalmology.

CONCLUSION

Transitions of care between secondary care settings are common for patients with IBD. Effective systems of data-sharing and care integration are essential to providing safe and effective care for patients. Geographic and age-related patterns of care transitions identified in this study may be used to guide interventions aimed at improving continuity of care.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Transitions of care; Continuity of care; Fragmentation; Multi-morbidity

Core tip: Patients with Inflammatory bowel disease (IBD) are often exposed to transitions of care between providers and settings which negatively impacts care continuity. This is the first paper to identify and measure the location and type of hospital encounters for IBD patients in England at a National level. Patterns of care identified in this study are important to guide the exchange of health information between providers to ensure safe, high quality care for patients with IBD.

Citation: Warren LR, Clarke JM, Arora S, Barahona M, Arebi N, Darzi A. Transitions of care across hospital settings in patients with inflammatory bowel disease. *World J Gastroenterol* 2019; 25(17): 2122-2132
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2122.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2122

INTRODUCTION

Inflammatory bowel disease

Inflammatory bowel disease (IBD) includes the chronic relapsing inflammatory disorders Crohn's disease and ulcerative colitis[1]. These are generally lifelong diseases, characterised by periods of remission and flares, with symptoms that include bloody diarrhoea, urgency, fatigue, weight loss, and abdominal pain. IBD affects 1 in 250 people in the United Kingdom giving an estimated prevalence of 240000[2]. The peak incidence occurs in patients between the ages of 15 and 30 years[3]. IBD may impact many aspects of the affected individual's life, accounting for substantial direct and indirect costs to the individual, the health care system and society[4].

Fragmentation of IBD care

A combination of factors including centralisation of healthcare services[5-7], difficulty accessing local services[8] and patient mobility between regions for education, employment or relationships may require IBD patients to access care in multiple settings. Furthermore, many IBD patients require care for extra-intestinal

manifestations of disease[9-12] which is often provided by several specialists in multiple settings. The unpredictable nature of disease may also require attendance to acute care services[13] in organisations separate to the patient's usual IBD care provider. The resulting multidisciplinary 'patient-sharing' between healthcare providers is characterised by multiple transitions of care. These transitions may impair continuity of care delivery and lead to care fragmentation[14].

Fragmentation of patient care is characterised by ineffective communication among providers and across healthcare agencies, insufficient patient and caregiver education, poor continuity of care, including medication reconciliation, and limited access to services, which contributes to negative quality and cost outcomes[15]. Fragmented inpatient care has been shown to be associated with a higher likelihood of in-hospital mortality, colonoscopy and longer readmission length of stay[16]. An increasing range of investigations and treatment options for IBD[17] adds further complexity to care transitions and necessitates the transfer of accurate and contemporaneous information at a secondary and tertiary care level.

Identifying transitions of care and patient-sharing in IBD

Quality standards in IBD care specify that services should be coordinated across the multidisciplinary care pathway[18]. Many patients, however, may still 'fall though the cracks' between providers[19]. The objective of this study was to determine the type and location of hospital services accessed by IBD patients in England. Identifying and measuring the frequency and distribution of patient-sharing may inform the development of more effective and efficient data-sharing practices between providers and assist in optimising systems at a local, regional and national level.

MATERIALS AND METHODS

This was a retrospective observational study using hospital administrative data. Adult patients resident in England that accessed inpatient care and had a recorded ICD-10 IBD disease-specific code (K50, K51) were identified from the Hospital Episode Statistics Admitted Patient Care dataset. Patients were recruited from this data set over a 2-year 'recruitment period' from April 2011 to March 2013. Each patient was then followed for a 2-year period from the date of their index admission, with the final patients recruited concluding follow-up by 30th March 2015. Patients that did not have any follow-up events after their index encounter were excluded from further analysis.

Identifying providers

In England, healthcare provider organisations, or 'Trusts', provide acute hospital services[20]. To accommodate organisational change over the study period, providers that merged or separated over the study period were treated as a single merged provider across the whole study period. Low-volume providers with less than 1000 total IBD patient encounters over the 4-year period of data were excluded.

Identifying 'home providers'

Each patient recruited into the study was allocated a 'home provider', which was identified as the Trust through which more of a patient's outpatient care in gastroenterology was delivered during the study period than any other provider. Patients that did not have any gastroenterology outpatient appointments were excluded from analysis.

Outcome measures

The primary outcome measure was the proportion of encounters that adult IBD patients in England have with their identified 'home provider'. Secondary outcome measures included the distribution of IBD patient-sharing, regional differences in IBD patient-sharing, age-related differences in accessing services and type and frequency of outpatient specialty services accessed by patients with a diagnosis of IBD.

Identifying frequency and location of healthcare events for IBD patients

We identified the frequency and location of accident and emergency, inpatient and outpatient encounters for IBD patients within National Health Service (NHS) England and determined the proportion of attendances to previously identified 'home providers'.

Identifying regional differences in patient events

Middle Layer Super Output Areas (MSOA) associated with each patient was used to map their residential region within England. MSOAs represent a geographic region

with a population between 5000 and 7200 people[21]. The estimates incidence of IBD in England is 0.5%-1%, yielding around 50 patients per MSOA[22]. To further analyse and illustrate regional differences in patient-sharing, the 20 provider organisations with the highest and lowest proportions of IBD patients attending their identified 'home provider' for healthcare were identified and mapped geographically.

Age-related differences in patient events

Access to care services was compared for three age bands, < 40, 40-70 and > 70 years, to determine differences in the proportion of patients accessing services through their 'home provider' for all patient encounter types.

Type of specialty services accessed by IBD patients

In NHS England, outpatient encounters are coded using main specialty codes or treatment function codes pertaining to the clinical service provided[23,24]. For recruited patients, we reviewed outpatient encounters within the follow-up period to determine the type of specialty services that IBD patients consulted with and the frequency of these.

Statistical methods

The investigators had complete access to the Hospital Episode Statistics (HES) dataset for the study period covering April 2011 to March 2015. Data was cleaned prior to analysis with removal of incomplete and duplicate records. Python (Python Software Foundation) was used for data extraction and analysis and Tableau (Tableau Software) for data visualisation. Statistical analysis and review were performed by biomedical statisticians (JC, MB).

RESULTS

Participants

126295 patients fulfilled the inclusion criteria and were recruited from the HES data set during the 2-year recruitment period. 31240 (24.7%) patients did not have gastroenterology appointments during the follow-up period and were therefore unable to be allocated a 'home provider' and excluded. 95055 patients remained for further analysis. This patient group had a total of 110300 accident and emergency, 304996 inpatient and 1344860 outpatient events over the 2-year follow-up period, including their first hospital admission through which they were recruited (Table 1).

Providers

76 low-volume providers with less than 1000 IBD patient encounters over the 2-year recruitment period were excluded, comprising a total of 8030 (0.00456%) encounters. A total of 144 providers remained for further analysis.

Frequency and proportion of 'home provider' encounters

1466155 of 1760156 (83.3%) IBD patient encounters were with the 'home provider'. Of those patients recruited who attended accident and emergency departments during the study period, 73.3% of those attendances were to their allocated 'home provider. 87.8% of inpatient hospital admissions in recruited patients were to their 'home provider' while 83.1% of outpatient attendances across all specialties were to their 'home provider' (Table 1). The range of proportions of 'home provider' encounters per trust was 37.0% to 94.3% for accident and emergency encounters, 57.2% to 98.5% for inpatient encounters and 55.7% to 96.9% for outpatient encounters.

Geographic distribution of IBD patient-sharing

There were regional differences in the proportion of 'home provider' encounters for each encounter type by the MSOA of residence of participants (Figure 1). For each 'home provider' the proportion of clinical encounters for patients allocated to that provider attending their 'home provider' was calculated. The highest and lowest 20 providers per proportion of 'home-provider' healthcare encounters is shown in Figure 2. Providers with a low proportion of 'home provider' encounters for IBD patients were typically located in metropolitan areas in Greater London and the North West of England and those with a high proportion of 'home provider' encounters were based outside major metropolitan areas.

Age-related differences in accessing care

The proportion of 'home provider' encounters for all event types in patients aged < 40, 40-70 and > 70 years illustrated in Figure 3. This shows lower 'home provider' encounters for patients under 40 years of age for all encounter types. The highest

Table 1 Inflammatory bowel disease patient encounters and proportion that were with the patient's 'home provider'

	Total encounters	'Home provider' encounters	'Home provider' proportion (%/% provider range)
Accident and emergency	110300	80836	73.3 (37.0-94.3)
Inpatient	304996	267540	87.8 (57.2-98.5)
Outpatient	1344860	1117779	83.1 (55.7-96.9)
Total	1760156	1466155	83.3

proportion of 'home provider' encounters was seen in the 40-70 years age group for all encounter types. All results were significant at $P < 0.001$ for pairwise $\chi 2$ tests.

Outpatient specialty services accessed by IBD patients

Specialty service and treatment codes pertaining to 130 different outpatient services were identified for included patients. These services included outpatient consultations and therapies, such as physiotherapy. The 20 most common outpatient medical and specialty services that IBD patients consulted with are listed in Table 2. Encounters with these 20 services constituted 84.3% of total outpatient events for IBD patients. There were 546768 gastroenterology outpatient appointments, accounting for 39.8% of all outpatient services accessed in this group of IBD patients. Between general surgery and colorectal surgery there were 96220 total outpatient encounters, accounting for 7.0% of outpatient encounters in this patient group. Ophthalmology consultations were also common, with 53237 (3.9%) encounters.

DISCUSSION

Through retrospective analysis of HES data we reviewed the records of 95055 patients with IBD and examined their interactions with NHS England hospitals over a 2-year period. These patients were involved in a total of 1760156 encounters during the 2-year follow-up period from recruitment. A majority of patients accessed accident and emergency, inpatient and outpatient care through the same 'home provider' that they attended for gastroenterology outpatient care. A substantial proportion of patients, however, accessed care from different hospital providers, particularly when using accident and emergency services (26.7% of accident and emergency encounters). This is an important finding that is congruent with previous research on the prevalence of fragmentation in IBD care[16] and underscores the need for effective systems to manage transitions of care and sharing of patient information between settings. Centralisation of care between hospitals is increasingly common in healthcare systems around the world and these findings may be replicated in other systems internationally. Poor interoperability of health record systems between organisations remains commonplace in many healthcare systems, including NHS England[25-28]. Primary care services traditionally aided in monitoring and guiding care coordination[29], however many patients in England find General Practitioner services difficult to access[8]. There is increasing momentum towards empowering IBD patients to take control of their own health records and disease management, although this requires infrastructure investment and may not be suitable for all patients[30]. Hospital providers therefore need to continue to improve interoperability or provide alternative effective data-sharing capacity to maintain continuity of care for patients using services across settings.

Regional differences in 'home provider' attendance

Analysis of the distribution of 'home provider' events by MSOA of participants and provider locations showed a trend towards increased non-'home provider' attendance in metropolitan centres. All of the 20 providers with the lowest proportion of IBD patients attending that same provider for healthcare were located in major metropolitan centres including London, Manchester, Birmingham and Liverpool. In these areas, the proportion of encounters with the usual gastroenterology 'home provider' was as low as 1 in 3 (37%) for accident and emergency encounters and only half of inpatient (57.2%) or outpatient (55.7%) encounters. Reasons for this may include increased service centralisation in these regions or ease of access to alternative providers for urgent or non-IBD related care. Regardless, this is an important finding as it indicates that within metropolitan centres, there is a more dynamic ecosystem of care and increased need to ensure adequate exchange of health information.

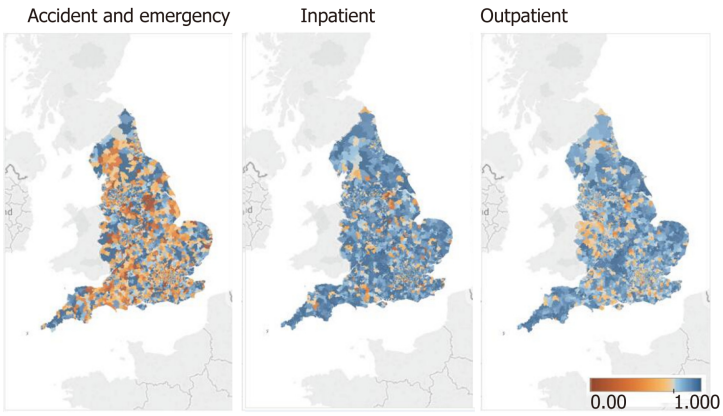

Figure 1 Proportion of accident and emergency, inpatient and outpatient presentations to inflammatory bowel disease care 'home provider' by Middle Layer Super Output Area of residence.

Accident and emergency events

More than one in four (26.7%) accident and emergency encounters were with a different hospital to the patient's gastroenterology 'home provider'. This is more than the proportion of non-'home provider' events for inpatient (12.2%) and outpatient (16.9%) services. Reasons for this finding may include a lack of accident and emergency services at the 'home provider' Trust, a need for urgent care necessitating presentation to the nearest hospital or patient preference. Importantly, this finding indicates that many patients seen acutely may not have comprehensive or up-to-date medical records held at that organisation. This may impact on the timeliness, effectiveness and safety of their care delivered by that provider. Additionally, information from an acute presentation may not be communicated with their usual 'home provider', again contributing to potential downstream transition of care errors.

It is also important to note that up to 19% of patients with IBD treated at a referral centre may be readmitted within 30 d[31]. Some patients may re-present to a different organisation than the previous provider, and these presentations may not be identified by those hospitals as readmissions. A lack of comprehensive, recent information regarding the patient may impact negatively on care and reduce the likelihood of avoiding preventable admission.

Transitions of care between specialty services

Improving communication and coordination between specialty services may reduce fragmentation of care and improve continuity for IBD patients. Specialty services accessed by IBD patients in this study reveals a broad range of services covering intraluminal and extraluminal disease. Clearly there is a need for effective information exchange between gastroenterology and general and colorectal surgical services with significant overlap between these specialties in the care of IBD patients. Previous studies estimate that approximately 10% of IBD patients experience eye problems such as uveitis, keratopathy, episcleritis and dry eyes[32,33] which may contribute in part to the frequent usage of ophthalmology services by patients in this study. Likewise, rheumatology and dermatology were some of the most common outpatient specialty services accessed by patients in this study and may reflect the increased predisposition to rheumatology and skin disease in IBD patients[10,34-36].

Age-related differences in care access

Some differences were seen in the proportion of 'home provider' care accessed by IBD patients across ages. Younger patients had a significantly lower proportion of care events with their 'home provider'. These differences were most prominent in accident and emergency encounters where patients under the age of 40 attended their 'home provider' for care on 70.5% of occasions, compared with 89.6% in patients aged 40-70 and 84.9% aged over 70. This may be explained, in part, by the increased mobility of younger patients who may be more likely to live, study or work in locations away from their 'home provider'.

Strengths and weaknesses of study

This was a retrospective observational study using a large, national administrative data set from 2013 to 2015. This has facilitated a novel analysis of transitions of care between secondary care settings for IBD patients in England. When applying these findings to the current population it is important to note that there may have been

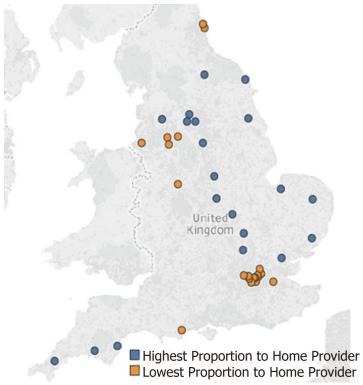

Figure 2 Distribution of 20 highest and 20 lowest providers per proportion of encounters with home provider (from 144 included providers).

changes to organisational structures and systems in addition to evolving regional demographics in the period since collection of this data. It is also important to note the inherent limitations of administrative data due to procedure changes, missing data and miscoding issues.

During patient recruitment and allocation of 'home providers' some losses may have resulted from the limitation of only being able to recruit patients from inpatient encounters. This approach was required as disease-specific codes are not allocated to outpatient and accident and emergency encounters within the data set. Additionally, patients that had existing but inactive disease also may not have been allocated an IBD disease code. In essence, only patients receiving care for 'active' IBD may have been recruited in some settings. This paper did not consider the reasons for the hospital events considered beyond the specialty responsible for that care event. This approach provided a clear overview of the services accessed but limited more in-depth interpretation of patient events, such as relevance of presentations to IBD and reasons for readmission.

Transitions of care between hospitals and primary care settings were beyond the scope of this study which used only hospital administrative data. Analysis of linked primary care and hospital-level databases may provide additional insights into hospital-primary transitions of care within this patient group and assist the important care coordination role played by many primary care providers. Patients under the age of 18 were excluded from analysis in this paper. This was necessary to permit an unbiased view of adult IBD patient-sharing. Although beyond the scope of this work, research to identify patterns of care transitions between paediatric and adult services using the methods developed in this paper may improve understanding of this challenging period for many young patients with IBD[37,38].

This paper has focussed on simple directed inter-organisational patient sharing connections. Previous, more complex healthcare network analysis studies have identified significant heterogeneity within patient sharing networks, with certain actors, whether hospitals or individual physicians, exercising different roles within a network[39-41]. More in-depth analysis of the networks studied in this paper may offer further insights into patient sharing within the NHS and further guide interventions. Additional analyses of other hospital-level factors such as hospital size, IBD patient numbers and IBD service availability may provide additional insights in future work. Furthermore, inclusion of existing data-sharing capacity between providers in a more complex analysis may provide additional value to guide future policy development.

Implications for providers and policy makers

The burden of disease for IBD patients can be reduced by improvements to care coordination and transitions of care between services. This study has shown that many patients with IBD in England access care from hospital providers in multiple settings. Younger patients and those residing in metropolitan areas tend to have their care shared between more providers and are at increased risk of transition of care errors in the absence of effective data-sharing practices. These groups are likely to benefit most from improvements to systems of health information exchange and care integration. Critically, this younger patient population may be more willing and able to adopt patient-led tools for medical record keeping, and therefore carry their clinical data with them on their mobile devices to be available to clinicians wherever they present. Similarly, improving transitions of care between specialty services such as gastroenterology, general and colorectal surgery, ophthalmology, trauma and

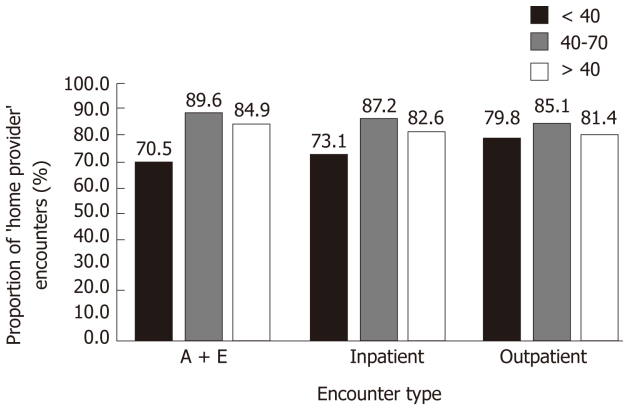

Figure 3 Proportion of 'home provider' encounters per age for three age bands, <40, 40-70 and > 70 years.

orthopaedics, rheumatology and dermatology are likely to benefit IBD patients. Organisations that regularly share IBD patients would benefit most from improved community data sharing. Further work to identify these patient-sharing networks and the important role of primary care services in these networks would assist in guiding improvements. The approaches used to identify hospitals and specialties that share the care of patents could be applied to other chronic and complex disease processes to better delineate provider care networks across systems.

In conclusion, to ensure quality and safe care for patients with IBD, providers should have access to the right information about the right patient at the right time. Findings from this work have shown that patients with IBD often transition between different hospital providers in multiple settings. This may act as a barrier to accessing up-to-date patient health information and negatively impact care. These findings should encourage and assist the development of mechanisms to enable effective and efficient coordination of care between providers that share the care of IBD patients.

Table 2 The 20 most frequently accessed outpatient services and corresponding proportion of total outpatient services

Outpatient service(HES Code)	Frequency (% of total services listed)
Gastroenterology (301)	546768 (39.8)
General surgery (100)	55126 (4.0)
Ophthalmology (130)	53237 (3.9)
Trauma and orthopaedics (110)	52510 (3.8)
Rheumatology (410)	50781 (3.7)
Colorectal surgery (104)	41094 (3.0)
Dermatology (330)	39392 (2.8)
Physiotherapy (650)	36895 (2.7)
General medicine (300)	32595 (2.4)
Cardiology (320)	29748 (2.2)
Diagnostic imaging (812)	29454 (2.1)
Urology (101)	27939 (2.0)
Gynaecology (502)	25260 (1.8)
Obstetrics (501)	22805 (1.7)
Respiratory medicine (340)	22238 (1.6)
Ear, nose and throat (120)	21953 (1.6)
Clinical haematology (303)	21031 (1.5)
Anticoagulant service (324)	18013 (1.3)
Nephrology (361)	14847 (1.1)
Clinical oncology (800)	14522 (1.1)

HES: Hospital Episode Statistics.

ARTICLE HIGHLIGHTS

Research background

Inflammatory bowel disease (IBD) is a chronic, inflammatory disorder characterised by both intestinal and extra-intestinal pathology. Patients may receive both emergency and elective care from several providers, often in different hospital settings. Poorly managed transitions of care between providers can lead to inefficiencies in care and patient safety issues. To ensure that the sharing of patient information between providers is appropriate, timely, accurate and secure, effective data-sharing infrastructure needs to be developed. To optimise inter-hospital data-sharing for IBD patients, we need to better understand patterns of hospital encounters in this group.

Research motivation

There is limited data on the types of hospital services accessed by patients with IBD and the frequency and location of hospital encounters. Identification of patterns of hospital care can guide inter-hospital data-sharing and care coordination which may improve continuity of care for these patients.

Research objectives

This study aimed to identify and quantify the hospital services accessed by patients with IBD in England.

Research methods

This retrospective observational study used Hospital Episode Statistics, a large administrative dataset in National Health Service in England, to identify characteristics of hospital care encounters for IBD patients. The proportion of encounters with providers other than the patients usual 'home provider' of IBD care was calculated, in addition to associations with patient age, location and type of specialist providers attended.

Research results

The proportion of encounters with hospitals other than the usual gastroenterology 'home provider' for 95055 IBD patients was up to 26.7% for accident and emergency encounters, followed by 16.9% for outpatient and 12.2% for inpatient encounters. Patients living in cities and younger patients were less likely to attend their 'home provider' for hospital services. The most commonly attended outpatient specialty services were gastroenterology, general surgery and ophthalmology.

Research conclusions

Up to one in four accident and emergency encounters for patients with IBD in England were with a different provider to the patient's usual gastroenterology 'home provider' of IBD care. IBD patients also often attended other hospitals for a range of outpatient and inpatient services. These findings emphasise the importance of developing effective data-sharing strategies between hospitals to maintain continuity of information and continuity of care for IBD patients.

Research perspectives

Findings from this study provide a national-level view of transitions of care between hospitals for patients with IBD in England. We have shown that certain groups of patients, including younger patients and those based in metropolitan areas, have more frequent transitions of care and may be a suitable target for further research and interventions to improve care continuity. Further qualitative and quantitative research is needed to understand the implications of these findings and improve inter-hospital data-sharing.

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World Journal of Gastroenterology

Submit a Manuscript: https://www.f6publishing.com

World J Gastroenterol 2019 May 7; 25(17): 2133-2143

DOI: 10.3748/wjg.v25.i17.2133

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Observational Study

Efficacy of Detoxsan® powder on diarrhea caused by gastrointestinal neuroendocrine tumors

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Author contributions: Langbein T, Dathe W, Deuerling A, and Baum RP contributed to study design; Langbein T and Baum RP contributed to patient recruitment; Dathe W and Deuerling A contributed to data collection, data analysis; Dathe W wrote the first draft of the paper; Langbein T and Baum RP contributed to critical revision of the paper; Baum RP contributed to final approval of article.

Institutional review board statement: The study was reviewed and approved by the scientific direction of the Theranostics Center for Molecular Radiotherapy and Molecular Imaging, Zentralklinik Bad Berka in Germany.

Informed consent statement: All study participants, or their legal guardians, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: The authors declare that they have no conflict of interest. Dathe W is an external advisor for Heck Bio-Pharma GmbH and licenser for Detoxsan®.

Data sharing statement: No additional data are available.

STROBE statement: The authors declare that the STROBE statement

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Abstract

BACKGROUND

Patients with neuroendocrine tumors (NETs) of the gastrointestinal tract suffer frequently from chronic diarrhea. A well characterized medical advice containing zeolite (Detoxsan® powder) was applied to patients suffered from therapy-refractory diarrhea either by its frequency or by watery stool, despite receiving standard pharmacotherapy according to the guidelines for carcinoid syndrome and comorbidities. Detoxsan® powder acts as an adsorbent and might reduce significantly symptoms of diarrhea in patients suffering from NETs.

AIM

To overcome the therapy-refractory diarrhea of patients with NETs by the zeolite containing medical advice Detoxsan® powder.

METHODS

A total of 20 patients (12 female and 8 male) suffering from diarrhea either by its frequency or from watery stool caused by NETs were included. In each patient, the diagnosis had been confirmed by histology and somatostatin receptors expression proven by positron emission tomography/computed tomography using Ga-68-labeled somatostatin analogs. All patients received standard-of-care pharmacotherapy and were additionally given Detoxsan® powder as an extemporaneous drug containing 90% natural Cuban zeolite and 10% magnesium aspartate. Recommended daily dosage ranges between 3 g once to three times per day. Each day dose and bowel movements were documented by the patients themselves in a pre-defined table. Additionally to the bowel movements quantitative determinations of serotonin, urea, creatinine and single ions were performed within the serum of the patients by commercially available equipment used as a matter of routine in the clinic.

was followed in the article entitled 'Efficacy of Detoxsan® powder on diarrhea caused by gastrointestinal neuroendocrine tumors' according to the checklist of items.

Manuscript source: Unsolicited manuscript

Received: November 25, 2018
Peer-review started: November 26, 2018
First decision: December 28, 2018
Revised: March 27, 2019
Accepted: March 29, 2019
Article in press: March 30, 2019
Published online: May 7, 2019

P-Reviewer: Capurso G, Cidon EU, Krishnan T
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

RESULTS

All patients enrolled in this pilot study did not only suffer from NETs, but also from comorbidities and treatment-resistant diarrhea. There was insufficient control of diarrhea, most probably due to the secretion of hormones like serotonin produced by the slowly growing and highly differentiated NETs. All patients only took Detoxsan® powder as an antidiarrheal drug. In general, response effects need several days to become perceptible and require an intake of Detoxsan® powder for an extended time period or intermittently, if persisting stabilization of bowel movements could not be achieved. A correlation between NET grade, part and size of bowel resection and functionality of the tumor could not be demonstrated. Therefore, diarrhea seemed to be based on the metabolic activity of the well-differentiated NETs, which eventually led to treatment resistance. In summary, 14 out of the 20 patients (70%) declared to be very content with using Detoxsan® powder and observed a significant reduction of diarrhea, while the effective dose and intake period that resulted in a symptom relief varied individually.

CONCLUSION

Detoxsan® powder is able to reduce significantly symptoms of NET-related diarrhea in the majority of patients. The duration of taking Detoxsan® powder and its dosage vary individually.

Key words: Neuroendocrine tumor; Diarrhea; Carcinoid syndrome; Treatment; Clinoptilolite; Mordenite; Zeolite

Core tip: A well characterized zeolite (Detoxsan® powder) was applied to patients with neuroendocrine tumors (NETs) of the gastrointestinal tract suffered from therapy-refractory diarrhea either by its frequency or by watery stool, despite receiving standard pharmacotherapy according to the guidelines for carcinoid syndrome and comorbidities. In 14 of 20 patients (70%) bowel movement rate could be normalized. Thus, Detoxsan® powder acts as an adsorbent and is able to reduce significantly symptoms of diarrhea in patients suffering from NETs. However, dose and period of intake have to be individually adjusted.

Citation: Langbein T, Dathe W, Deuerling A, Baum RP. Efficacy of Detoxsan® powder on diarrhea caused by gastrointestinal neuroendocrine tumors. *World J Gastroenterol* 2019; 25(17): 2133-2143
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2133.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2133

INTRODUCTION

There are diverse etiologies of diarrhea due to different or tissue-dependent mechanisms[1]. Chronic diarrhea can be caused by neuroendocrine tumors (NETs) of the gastrointestinal tract (previously called "carcinoids"), which lead to significantly reduced quality of life. Application of synthetic analogues of somatostatin[2] is one of the established treatment options to control characteristic symptoms like flushing and diarrhea as well as to inhibit tumor growth. A significant proportion of patients might stop responding to somatostatin analogue therapy or this treatment alone might not achieve adequate symptom relief. Therefore, new and active substances are certainly required to alleviate relevant symptom burden like diarrhea. A prospective phase III clinical study using Telotristat Etiprate (Xermelo™), a novel serotonin synthesis inhibitor, reported a significant decrease in daily bowel movements frequency, decrease in serotonin production and increase in quality of life[3,4]. Telotristat Etiprate is a tryptophan hydroxylase inhibitor, and thereby reduces the production of serotonin and diminishes daily bowel movements. Since 2017, Xermelo™ is approved in by the FDA as well as by EMA for the treatment of carcinoid syndrome-related diarrhea in combination with somatostatin analog therapy[5]. It implies that the most

recently approved drug is based on the inhibition of the production of serotonin.

Serotonin or 5-hydroxytryptamine (5-HT) is well known as the cerebral 'hormone of happiness', while the enteric 5-HT is a polyfunctional signaling molecule, acting as a paracrine factor, an endocrine hormone or a growth factor, which is important in gastrointestinal motility, enteric neurogenesis and intestinal inflammation[6]. Approximately 95% of 5-HT is synthesized in the gastrointestinal tract, stored in mucosal enterochromaffin cells and released by mechanical and chemical stimulations[7]. Spohn and Mawe[8] could show that bacteria within the lumen of the bowel influence serotonin synthesis and release by enterochromaffin cells. Furthermore, colon epithelial cells directly exposed to serotonin are primed for inflammatory reactions. Thus, the elevated serotonin level seems to be an important innate immune response, induces inflammatory genes in the gut and may be responsible at least partially for 5-HT-mediated pathogenesis in patients with inflammatory bowel disease[9]. On the other hand, the amino acid tryptophan acting as the precursor of both serotonin and niacin (vitamin B3), which may induce niacin deficiency by uncontrolled serotonin production as reviewed[10]. The clinical complex appearance of NETs – the so called "carcinoid syndrome" – is most frequently based on serotonin overproduction and involves severe diarrhea and flushing as well as bronchial obstruction, wheezing muscle wasting as well as proximal myopathy and may lead to carcinoid heart disease consisting of a secondary tricuspid valve insufficiency due to endocardial fibrosis[10]. Thus, serotonin downregulation within the gastrointestinal tract might cause reduction of bowel movements.

Natural zeolites are characterized by attractive properties such as adsorption, ion-exchange and molecular sieving. Due to the lattice structure of the aluminosilicates with channels and cavities, they possess an excellent binding capacity for ions, toxins and other harmful substances, which privilege them for medical and biomedical applications. Therefore, they are widely used in dietary supplements, as active ingredients in drugs or carriers for drugs, adjuvants in anticancer therapy and several other applications[11-15]. In particular, the natural zeolite clinoptilolite has been traditionally used in a large number of biomedical applications, due to its physico-chemical stability and biological compatibility. It has proven to be an effective anti-diarrheic drug[16]. Furthermore, a majority of the patients suffering from irritable bowel syndrome with diarrhea responded effectively to artificially enhanced clinoptilolite[17]. The natural Cuban zeolite used in this study is already available in Germany as an extemporaneous mixture (Detoxsan® Pulver) and is composed of two types of zeolite structures having different pore sizes: clinoptilolite, a medium-pore 10-membered ring zeolite and mordenite, a large-pore 12-membered ring zeolite[18]. Moreover, this Cuban zeolite is able to adsorb remarkable amounts of the biogenic amine histamine and water[18-20]. It has been applied for the first time to patients suffering from severe diarrhea caused by NETs. In diarrhea related to medullary thyroid cancer, montmorillonite clay had been applied successfully in 10 patients as a pilot study[21]. Montmorillonite clay belongs to the layered aluminium silicates while zeolites used in this study are characterized by 3-dimensional crystal lattices with different characteristics[22,23].

MATERIALS AND METHODS

Patients

In the present study, a total of 20 patients (12 female and 8 male) suffering from diarrhea either by its frequency or from watery stool caused by NET were included, age ranged from 39 and 83 years (mean age of 64.1 years) (Table 1). In each patient, the diagnosis had been confirmed by histology and somatostatin receptors expression proven by positron emission tomography/computed tomography using Ga-68-labeled somatostatin analogs[24]. All patients received standard-of-care pharmacotherapy (Table 2) and were additionally given Detoxsan® powder (Detoxsan® Puder) as an extemporaneous drug containing 90% natural Cuban zeolite (clinoptilolite and mordenite) and 10% magnesium aspartate. Intake of Detoxsan® powder was commenced with low doses of 3 × 1 g/d or 2 × 2 g/d and increased up to 3 × 3 g/d or 3 to 5 g every 4 h if necessary, as recommended in the literature about common non-infectious diarrhea for few days only[16]. Individually tailored dose of Detoxsan® powder will be mentioned in detail in the 'Results' section. Each day dose and bowel movements were documented by the patients themselves in a pre-defined table.

Biochemical determinations

Serum serotonin levels were measured by a commercially available ELISA kit

Table 1 Patient cohort

Patient	Female	Male	Age (yr)	Grading	Functionality	Resection (cm)	HBP	Others
1	X		55	G2	yes, WD	IL, 62 + CC, 4		X
2		X	68	G2	yes, WD	IL, 40	X	X
3	X		72	G2	yes, WD	IL, 56 + CO, 11	X	X
4	X		79	G1	yes, WD	ileocecal		X
						IL + jejunum, 150		
5	X		60	G1	yes, WD	hemicolectomy	X	X
6	X		65	G2	yes, WD	hemicolectomy		X
7	X		47	G2	yes, WD	hemicolectomy, 220		X
8		X	77	G1	yes, WD	hemicolectomy, 120		X
9	X		43	G2	no, WD	no		X
10		X	61	G2	MD, Serotonin	ileocecal, 40		X
11	X		73	G1	yes, WD	hemicolectomy, IL		X
12		X	57	G1	yes, WD	mid IL		X
13		X	63	G2	yes, WD	IL segments	X	X
14	X		48	G2	yes, WD	IL, 23 + CO, 31	X	X
15		X	64	G2	yes, WD	IL, 70	X	
16		X	77	unknown	WD, Chromogranin	IL, 64		X
17	X		74	G2	yes, WD	IL, 18		X
18	X		78	unknown	yes, WD	no	X	X
19		X	83	G1	Chromogranin	no		X
20	X		39	G1	no, well diff.	no	X	X
Total	12	8						
Average age	61.1	68.7	64.1					

Grading: Of neuroendocrine tumors according to WHO classification[29]; WD: Well-differentiated neuroendocrine neoplasm; MD: Moderately-differentiated neuroendocrine neoplasm; CC: Coecum; CO: Colon; IL: Ileum; HBP: Hypertension or high blood pressure.

(Serotonin FAST ELISA, DRG Instruments GmbH, Marburg, Germany) according to instructions given by the company. Incubation was performed at room temperature for 15 min and the antigen antibody complex containing serotonin was determined at 450 nm. Urea had been quantified by a kinetic assay using urease and glutamate dehydrogenase. In the first step urea is split by urease into ammonia and in the second enzymatic reaction the released ammonia forms L-glutamate from 2-oxoglutate using NADH. The rate of decrease of NADH measured at 340 nm is directly proportional to the concentration of urea in the assay[25]. Determination of single ions was performed by ion-selective electrode (ISE) using commercially available equipment (Tecan's Sunrise absorbance microplate reader). The electrochemical sensors are helpful tools for qualitative and quantitative ion measurements[26]. Therefore, ISE serves as the standard method in our laboratory.

RESULTS

All patients enrolled in this pilot study did not only suffer from NETs, but also from comorbidities and treatment-resistant diarrhea (Table 1). They took Detoxsan® powder as an antidiarrheal drug, moreover, the intake of loperamide, which is a commercially available antidiarrheal drug, was recommended as well (See Table 2). Nevertheless, in the predefined table documented by the patients themselves, we could not found the intake of loperamide by the patients.

The individual dose of Detoxsan® powder was adapted by patients themselves with an increase until a significant reduction of bowel movements was reached (Figure 1) or a decrease if bowel movements frequency declined (Figure 2). In general, response effects need several days to become perceptible and require an intake of Detoxsan® powder for an extended time period or intermittently, if persisting stabilization of bowel movements could not be achieved (Figure 3). However, the use of Detoxsan® powder did not satisfy every patient or could reduce bowel movements (Tables 3 and 4). Three patients could not benefit even at a higher concentration and stopped daily intake ahead of schedule. Three patients reached only partial reduction of diarrhea. In

Table 2 Clinical parameters of the patients

Patient	Nutricion	Somatostatin	Dosage	Interval	Loperamide[1]	Disease	NET type	Primary	Metastases in				PRRT
	consultation	analog				stage			Liver	Lymph	Bone	Lung	Peritoneum	
1	yes	OCT	30 mg	4 wk	if required	IV	functional	midgut	x	x			x	2nd
2	yes	OCT	20 mg	4 wk	if required	IIIb	functional	midgut	x	x			x	3rd
3	yes	OCT	30 mg	4 wk	no	IV	functional	midgut	x	x	x		x	2nd
4	yes	LAN	120 mg	6 wk	no	IV	functional	midgut	x	x	x	x		2nd
5	yes	LAN	120 mg	3 wk	no	IV	functional	midgut	x		x			2nd
6	yes	OCT	30 mg	4 wk	no	IIIb	functional	midgut	x	x				4th
7	yes	OCT	30 mg	4 wk	no	IIIb	functional	midgut	x	x				3rd
8	yes	LAN	120 mg	4 wk	if required	IV	functional	midgut	x	x				2nd
9	yes	no			no	IV	nonfunctional	pancreas	x	x	x			1st
10	yes	OCT	30 mg	4 wk	no	IV	functional	midgut	x	x	x			3rd
		OCT s.c.	+ 100 μg	if required										
11	yes	OCT	30 mg	4 wk	no	IIIb	functional	midgut	x				x	1st
12	yes	LAN	120 mg	4 wk	if required	IV	functional	midgut		x				2nd
13	yes	OCT	30 mg	4 wk	if required	IV	functional	midgut	x					3rd
14	yes	LAN	120 mg	4 wk	no	IV	functional	appendix		x				2nd
15	yes	OCT	30 mg	4 wk	no	IV	functional	midgut	x	x			x	3rd
16	yes	OCT	20 mg	4 wk	if required	IV	functional	midgut	x				x	2nd
17	yes	LAN	120 mg	4 wk	if required	IIIb	functional	midgut	x	x	x			2nd
18	yes	OCT	30 mg	3 wk	if required	IV	functional	CUP	x	x				3rd
19	yes	OCT	30 mg	4 wk	no	IV	nonfunctional	pancreas	x		x		x	1st
20	yes	OCT	30 mg	4 wk	no	IV	functional	CUP	x	x				3rd

[1]During treatment with Detoxsan® powder. OCT: Octreotide (Sandostatin LAR); LAN: Lanreotide (Somatuline Autogel); CUP: Unknown primary with suspicion of midgut primary; PRRT: Peptide receptor radionuclide therapy as treatment line. Determination of the disease stage according to the WHO classification[29].

general, response rates of Detoxsan® powder appears to correlate with patient's nutrition, *e.g.* raw salad, fatty food and sauerkraut were reported to have negative effects on diarrhea despite intake of the powder.

In summary, 14 out of the 20 patients (70%) declared to be very content with using Detoxsan® powder and observed a significant reduction of diarrhea, while the effective dose and intake period that resulted in a symptom relief varied individually (Table 3). Moreover, some patients stopped oral application when bowel movement became regular, whereas other patients extended intake in order to keep bowel movements at a low tolerable level. Apparently, that individual decision seemed to be dependent on the tolerance of patients with the number of bowel movements and stool consistency, in which a variance could be observed. A correlation between NET grade, part and size of bowel resection and functionality of the tumor could not be demonstrated (Tables 1-3). Therefore, diarrhea seemed to be based on the metabolic activity of the well-differentiated NETs, which eventually led to treatment resistance.

The serum serotonin level appears to be one of the major factors responsible for diarrhea in NETs[3-5], which is why blood serotonin level was determined and recorded on follow-up (Table 5). Only in 6 patients (patients 4, 5, 10, 14, 19, 20), serotonin level was measured in a short time interval before and after intake of Detoxsan® powder. Independent of the potential wide range of level of this biogenic amine, there was a significant decrease in the serotonin level during the period of intake in all that cases. To evaluate if other commonly detected laboratory parameters are affected by Detoxsan® powder intake, we determined exemplarily the levels of creatinine and urea as well as the cations sodium (Na), potassium (K) and calcium (Ca) in the blood (Table 5). In the creatinine and urea levels only a slight increase was measured during the use of Detoxsan® powder while the investigated electrolytes did not exhibit any significant changes of their level.

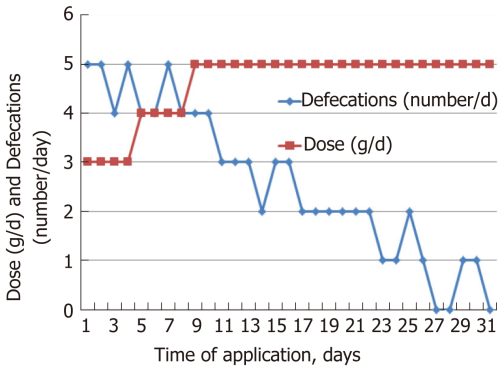

Figure 1 Application of Detoxsan® powder and frequency of bowel movement of a 77-year-old male patient.

DISCUSSION

All the patients enrolled in this pilot study did not only suffer from NETs, but also from comorbidities and treatment-resistant diarrhea due to reduced resorption capacity of the bowl with a consequent intestinal failure (Table 1)[27,28]. Grading and differentiation of NETs were categorized according to the recent WHO classification[29]. Length of bowel resection was included in the characterization of patient status. Principally, patients were treated according to the Theranostic concept for NETs based on national and international guidelines[24]. However, there was insufficient control of diarrhea, most probably due to the secretion of hormones like serotonin produced by the slowly growing and highly differentiated NETs[2].

Whether the serotonin level decrease in 6 patients is based on the adsorption of serotonin by the zeolite – similar to the histamine uptake – or the decline of the chemically-labile serotonin is caused by other mechanisms is still the subject of ongoing investigations[18,19]. Nevertheless, these 6 patients were part of the satisfied ones with respect to the bowel movements (Table 2) corresponding to correlation of serotonin level and diarrhea[3-5]. For further investigations it is worth to determine the level of 5-hydroxyindoleacetic acid in a 24-h urine collection. It is a stable metabolite of serotonin and by this way its level can be determined indirectly[30]. It was not done in our present research.

Despite the known facts that enteric serotonin is a polyfunctional signaling molecule, an essential component of the gastrointestinal inflammatory response and a bioactive component in developing and mature animals, the effect of this amine on diarrhea is still not comprehensively understood[6]. In weaning mice *e.g.*, stress-induced diarrhea is considered to be caused by deregulation of the mucosal immune system (among others). Interestingly, mucosal immunity was decreased in the duodenum and jejunum without being affected in the ileum and colon[31,32]. Given the fact that the intestinal tract is the largest immune organ of the human body, serotonin might be therefore considered to be a link between the gut and the immune regulation[33,34]. Moreover, histamine is one of the most important biogenic amines and strongly involved in immunological reactions. It has been described as 'an undercover agent in multiple rare diseases', because many pathological inflammatory processes are involved with histamine as well[35].

The positive effect of zeolite on treatment-resistant diarrhea caused by NETs might be related to several origins. High adsorption capacity for histamine and possibly other biogenic amines like serotonin, the antiphlogistic effect of this mineral as well as its high water uptake capacity are potentially responsible for reducing diarrhea complaints[18-20]. This kind of treatment differs significantly from all other drugs in the field because Detoxsan® powder acts only *via* its inherent adsorption properties within the gastrointestinal tract; neither does it penetrate into the blood circulation nor directly influence regulation processes. Furthermore, it is noteworthy that the histamine uptake of the Cuban zeolite (and possibly of other biogenic amines) is significantly higher compared to other zeolites containing only clinoptilolite and no additional mordenite[19]. The clinical treatment of patients with NETs with regard to their functional complaints is recently focused to the application of somatostatin receptor inhibitors[36]. Thus, the aim of both methods appears similar while the pharmacological approach is quite different. The slight increase of the creatinine and urea levels during the use of Detoxsan® powder might be interpreted as a mild decrease in the kidney filtration process while the metabolic liver function seems to be unaffected.

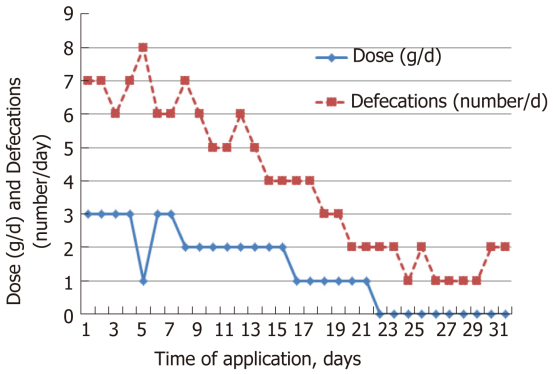

Figure 2 Application of Detoxsan® powder to a 64-year-old male patient.

In conclusion, the contemporaneous mixture Detoxsan® powder is able to reduce significantly symptoms of NET-related diarrhea in the majority of patients. The effect of this zeolite seems to be due to its high capacity to bind water, histamine and possibly serotonin, too, within the gastrointestinal tract and to removal of those compounds *via* stool. The duration and dose of Detoxsan® powder intake varies individually. In some patients the normalization of bowel movement could be observed within a few weeks while some patients need to use it permanently in order to maintain acceptable quality of life without diarrhea. Recommended daily dosage requires an individual adaptation and ranges between 3 g once to three times per day. The clinical reduction of the diarrhea symptoms by Detoxsan® powder comes without a relevant negative influence on other biochemical parameters.

Table 3 Effect of Detoxsan® powder on the bowel movements as evaluated by patients

Patient	Empirical evaluation of the patients			Intake time (d)	Dose (g/d)	Bowel movement
	Satisfied	Uncertain	Non-satisfied			(frequency per day)
1			X	41	4 x 5	9 to 12
2			X	10	6 x 3	8 to 11
3	X			160	1 x 3	1 to 3
4		X		118	3 x 3	2 to 3
5	X			201	3 x 3	3 to 5
6		X		106	3 x 3	1 to 3
7	X			215	2 x 2	3
8	X			605	2 x 2	1 to 3
9	X			21	3 x 1	1 to 2
10	X			134	2 x 2	3 to 5
11	X			220	3 x 1	2 to 3
12	X			427	2 x 3	2 to 3
13	X			138	2 x 2	1 to 2
14	X			31	2 x 3	2 to 4
15	X			21	1 x 3	1 to 2
16	X			31	1 x 5	1
17			X	20	2 x 3	6 to 7
18		X		62	2 x 3	3 to 4
19	X			31	1 x 3	1 to 2
20	X			31	2 x 3	1 to 3
Total	15	3	3			
Percentage	70.0	15.0	15.0			

Responses were categorized as: Satisfied-bowel movements significantly reduced, uncertain-bowel movements not permanently reduced and non-satisfied-no improvement of defecation number.

Table 4 Diary of a 57-year-old male patient using Detoxsan® powder over 3 mo

Detoxsan® powder intake (d)	days	Dose (g/d)	Bowel movements (frequency per day)	Empirical evaluation, daily	
				Satisfied	Non-satisfied
1 to 2		2 x 2	5 to 8		2
	3 to 8	2 x 2	1 to 4	6	
9 to 11		2 x 2	5 to 8		3
12 to 13		2 x 3	5 to 8		2
	14 to 26	2 x 2	1 to 4	13	
27 to 28		2 x 2	5 to 8		2
	29 to 31	2 x 2	1 to 4	3	
	32 to 43	2 x 3	1 to 4	12	
44 to 49		2 x 3	5 to 8		6
	50 to 75	2 x 3	1 to 4	26	
76 to 79		2 x 3	5 to 8		4
	80 to 87	2 x 3	1 to 4	8	
88 to 91		2 x 3	5 to 8		4
	92 to 120	2 x 3	1 to 4	28	
Sum of days				96	23

The dose of Detoxsan® powder was individually adjusted. The treatment effect was assessed by the patient himself.

Table 5 Blood parameters determined before (in brackets) and after the application of Detoxsan® powder

Patient	Serotonin (µg/L)	Creatinine (µmol/L)	Urea (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Calcium (mmol/L)
1	(1.554) 2.442	(58.0) 62.4	(3.1) 2.7	(139) 134	(3.7) 5.2	(2.3) 2.2
2	(ND) 940	(126.7) 142.3	(5.5) 7.3	(139) 142	(4.0) 4.2	(2.4) 2.4
3	(289) 530	(59.0) 69.8	(4.1) 5.2	(138) 133	(4.9) 4.4	(2.3) 2.2
4	**(919) 718**	**(75.0) 92.1**	**(4.0) 4.8**	**(134) 140**	**(3.9) 4.0**	**(2.2) 2.3**
5	**(1.834) 1.330**	**(57.9) 91.9**	**(4.4) 5.4**	**(144) 145**	**(4.2) 3.9**	**(2.3) 2.3**
6	(398) 540	(72.8) 91.8	(7.8) 10.4	(141) 137	(4.3) 4.1	(2.0) 2.1
7	(ND) 318	(53.0) 46.0	(3.1) 3.1	(139) 139	(3.9) 3.4	(2.3) 2.1
8	(946) 399	(167.5) 204.7	(4.7) 9.9	(142) 145	(4.2) 5.0	(2.3) 2.1
9	(394) 575	(73.2) 66.9	(3,7) 5.1	(136) 138	(4.3) 4.4	(2.3) 2.3
10	**(2.500) 688**	**(60.5) 83.7**	**(3.6) 3.8**	**(146) 142**	**(3.8) 4.5**	**(2.3) 2.3**
11	(263) 1.939	(59.3) 93.8	(6.8) 6.5	(144) 142	(4.3) 4.2	(2.1) 2.0
12	(1.102) 1.025	(85.6) 70,6	(4.1) 3.4	(143) 142	(3.9) 3.2	(2.2) 2.2
13	(1.230) 708	(70.0) 69.5	(5.4) 3.5	(144) 145	(4.0) 4.3	(2.4) 2.5
14	**(1.967) 775**	**(46,9) 54,9**	**(2.6) 3.2**	**(142) 143**	**(4.4) 4.3**	**(2.3) 2.3**
15	(2.052) 2.500	(75.1) 110.7	(4.6) 6.3	(137) 141	(3.9) 3.8	(2.3) 2.3
16	(477) 353	(58.6) 55.9	(5.8) 7.0	(144) 146	(4.2) 4.8	(2.2) 2.4
17	(1.224) 1.446	(50.3) 48.3	(3.5) 3.9	(143) 142	(4.0) 4.0	(2.3) 2.3
18	(1.185) 2.500	(52.7) 61.6	(3.5) 3.4	(143) 140	(4.2) 4.3	(2.3) 2.3
19	**95 (63)**	**(155.0) 171.5**	**(8.2) 13.9**	**(133) 137**	**(4.1) 4.6**	**(2.3) 2.3**
20	**68 (64)**	**(75.5) 74.3**	**(5.3) 5.8**	**(144) 142**	**(4.5) 4.1**	**(2.4) 2.4**

Bold values indicate that parameters were determined within a short time interval (< 30 d) before and after using Detoxsan® powder, respectively.

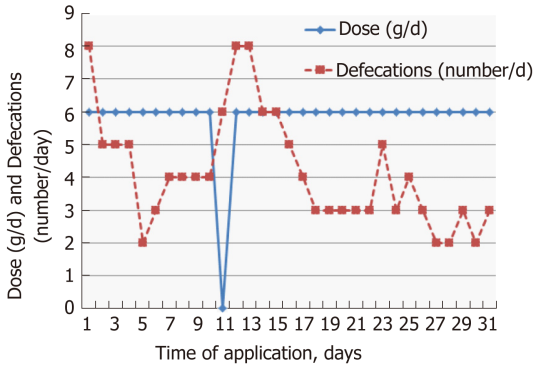

Figure 3 Application of Detoxsan® powder to a 63-year-old male patient.

ARTICLE HIGHLIGHTS

Research background

Therapy-refractory diarrhea in neuroendocrine tumor (NET) patients reduces quality of life, strongly restricts their daily routine and is therefore a highly clinical unmet need.

Research motivation

Motivation of this investigation was reduce bowel movements in chronic diarrhea patients and by this to achieve a significant improvement in their quality of life.

Research objectives

To overcome the therapy-refractory diarrhea of patients with NETs by the zeolite containing medical advice Detoxsan® powder.

Research methods

For this purpose, patients were offered a well characterized zeolite product which is known to adsorb biogenic amines and water in large extent and it does not enter into the blood stream. The patients have been informed in detail about the product, the individual adaptation of the dosage and the documentation in a predefined table. Due to the fact that diarrhea syndrome is a

disturbance of the daily routine the patients enrolled in this clinical trial respected these recommendations and showed an excellent compliance. In addition to the clinical trial, we determined some biochemical parameters in order to monitor if some undesired changes occur.

Research results

It is the first time that a well characterized zeolite product is able to reduce bowel movements in patients suffered by therapy-refractory diarrhea caused by NETs over a long time. In 14 of 20 patients (70%) enrolled in the trial bowel movement rate could be normalized. The application time to reach an acceptable bowel movement oscillated between few weeks up to a permanent or intermittent use. Also the dosage oscillated between 3 g per day up to three times 3 g per day. All of the 14 responder patients appreciated the normalization of the bowel movements in spite of the individual adaptation of dosage and time. There were no side effects. However, it is not clear which factors influence the reduction of diarrhea. At least one component namely serotonin seems to be involved in this physiological process. Therefore, the adsorption of this biogenic amine by this type of zeolite is under investigation. Furthermore, the serotonin metabolite 5-hydroxyindoleacetic acid should be determined in a 24-h urine collection. Using this method the natural serotonin secretion can be determined indirectly.

Research conclusions

The new finding of this study is the effective application of a well characterized zeolite product in patients suffered by therapy-refractory diarrhea caused by NETs. The attractive properties of the lattice structure of this mineral for excellent binding capacity for water, amines and harmful substances seem to possess a key function in overcome diarrhea symptoms in both temporary application and long term use. The individual dosage and period of application in order to receive the best reduction of diarrhea indicate that the physiological process of these symptoms is not fully understood and requires further investigations. For the clinical practice it is important to accept the individuality of this treatment to overcome patient's diarrhea and improve their daily routine.

Research perspectives

We observed that a well characterized natural zeolite is able to overcome therapy-refractory diarrhea caused by NETs *via* passing the gastrointestinal tract only. However not all effects could be answered satisfactorily. Therefore, future research should be focused on the one hand to the adsorption of serotonin and other trigger substances for diarrhea by this zeolite. On the other hand the clinical treatment of NETs patients requires the determination of the biochemical factors in both blood and faeces in correlation the resected bowel part and the effect of this zeolite. The aim of these investigations should be the selective dosage of the mineral product on the basis of biochemical values and/or surgical data.

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World Journal of Gastroenterology

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World J Gastroenterol 2019 May 7; 25(17): 2144-2148

DOI: 10.3748/wjg.v25.i17.2144

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Tuberculous esophagomediastinal fistula with concomitant mediastinal bronchial artery aneurysm-acute upper gastrointestinal bleeding: A case report

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Author contributions: Alharbi SR involved in diagnosis and treatment of the patient and after that review the literature and write the manuscript.

Informed consent statement: Informed consent was obtained from the patient for the publication of the report.

Conflict-of-interest statement: The authors have declared no conflicts of interest.

CARE Checklist (2016) statement: The authors have read the CARE Checklist (2016), and the manuscript was prepared and revised according to the CARE Checklist (2016).

Manuscript source: Unsolicited manuscript

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Abstract

BACKGROUND

Esophagomediastinal fistula is a very rare complication of tuberculosis in otherwise healthy adults, and mediastinal bronchial artery aneurysm is even rarer. In this case report, we describe a rare case of tuberculosis complication that presented with acute upper gastrointestinal (GI) bleeding. It also highlights the benefits of chest computed tomography (CT) as an excellent adjunct diagnostic tool to endoscopy and bronchoscopy and the role of trans-arterial embolization as a minimal invasive therapy alternative to surgery.

CASE SUMMARY

A 19-year-old medically free male patient presented with acute multiple episodes of hematemesis for 1 d. Upper GI endoscopy, bronchoscopy, and chest CT with IV contrast confirmed esophagomediastinal fistula with mediastinal bronchial artery aneurysm. After resuscitating patient with IV fluid and blood product transfusion, trans catheter embolization was performed for mediastinal bronchial artery aneurysm.

CONCLUSION

We successfully treated a patient with acute upper GI bleeding due to tuberculous esophagomediastinal fistula and mediastinal bronchial artery aneurysm using transcatheter coil embolization.

Key words: Mediastinal bronchial artery aneurysm; Esophagomediastinal fistula; Upper gastrointestinal bleeding; Mediastinal tuberculosis; Case report

Received: February 16, 2019
Peer-review started: February 18, 2019
First decision: March 14, 2019
Revised: March 29, 2019
Accepted: April 19, 2019
Article in press: April 20, 2019
Published online: May 7, 2019

P-Reviewer: Lu XL, Nagliati C
S-Editor: Yan JP
L-Editor: A
E-Editor: Ma YJ

Core tip: A 19-year-old medically free male patient presented with acute upper gastrointestinal bleeding. He underwent endoscopy, bronchoscopy, and chest computed tomography with IV contrast. Diagnosis of pulmonary and mediastinal tuberculosis with esophagomediastinal fistula and mediastinal bronchial artery aneurysms was made. Patient was successfully treated with mediastinal bronchial artery aneurysm coil embolization and antitubercular medications.

Citation: Alharbi SR. Tuberculous esophagomediastinal fistula with concomitant mediastinal bronchial artery aneurysm-acute upper gastrointestinal bleeding: A case report. *World J Gastroenterol* 2019; 25(17): 2144-2148
URL: https://www.wjgnet.com/1007-9327/full/v25/i17/2144.htm
DOI: https://dx.doi.org/10.3748/wjg.v25.i17.2144

INTRODUCTION

Mediastinal tuberculosis (TB) lymphadenitis is a rare clinical manifestation of TB in adults, and it usually occurs secondary to pulmonary TB. Extrapulmonary lymph node TB was reported to be 17.6% of all TB cases[1]. Esophagomediastinal fistula formation secondary to mediastinal TB lymphadenitis is an unusual complication that develops as a consequence of erosion of adjacent organs[2]. Mediastinal bronchial artery aneurysms are extremely rare and can be idiopathic or secondary. Secondary aneurysms are usually related to an underlying inflammatory process like bronchiectasis, chronic bronchitis, or systemic vascular disease[3,4]. Bronchial artery aneurysm with an esophageal fistula is an extremely rare and potentially fatal diagnosis, and there are few case reports in literature[5].

CASE PRESENTATION

A 19-year-old medically free patient presented to our emergency department with 1-d history of 5 episodes of hematemesis with moderate amount associated with epigastric pain. His medical history is significant for chronic cough with occasional hemoptysis and weight loss for 5 mo. His vital signs are stable. Patient was pale, but there was no jaundice or cyanosis. Chest and abdominal examinations are unremarkable. Laboratory investigations show normal results except low hemoglobin level of 8 g/dL. Two units of packed red blood cells (PRBCs) were transfused. Upper gastrointestinal (GI) endoscopy shows an opening in the mid-esophagus without clot, bleeding, or mucosal lesions, otherwise a normal esophagus, stomach, and duodenum (Figure 1). Tracheoesophageal fistula was suspected, and no definite source of bleeding was identified.

Bronchoscopy was performed and showed no tracheoesophageal fistula but edematous mucosa with nodular lesions in the left lower lobe bronchus. Biopsy was performed. Initial chest X-ray shows cavitary left lung lesion. Three sputum samples were sent for Ziehl-Neelsen (ZN) staining and were negative. Chest computed tomography (CT) with IV contrast was performed and showed evidence of esophagomediastinal fistula, multiple necrotic mediastinal lymph nodes, cavitary lung lesion, and two small mediastinal bronchial artery aneurysms indicating pulmonary and mediastinum TB (Figure 2). Patient had another episode of hematemesis with moderate amount. Upper GI endoscopy was repeated and unremarkable except a small opening in the mid-esophagus. Another two units of PRBC were transfused. Conventional angiography was performed and showed bronchial artery arising from the left subclavian artery with two small mediastinal bronchial artery aneurysms. Embolization was performed using coils (Figure 3). Histopathological results of bronchial biopsy showed necrotizing granulomatous inflammation suggesting TB, and two additional sputum samples were sent for ZN staining and were positive. Anti-TB medication was started. Patient did not have hematemesis or melena after embolization. Patient was discharged in good condition with anti-TB medications. At 3-mo of follow-up, patient was asymptomatic and had no episode of GI bleeding. Follow-up chest X-ray shows interval improvement (Figure 4).

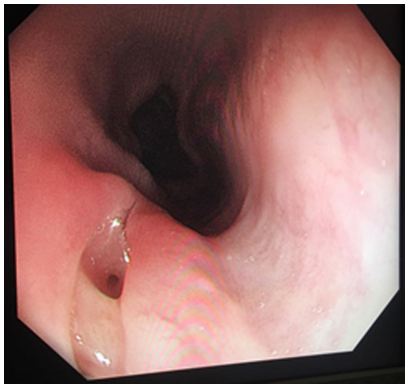

Figure 1 Endoscopy image: An opening is seen in the mid-esophagus without active bleeding or clots.

FINAL DIAGNOSIS

Tuberculous esophagomediastinal fistula with mediastinal bronchial artery aneurysm.

TREATMENT

Patient was treated by mediastinal bronchial artery aneurysm trans catheter coil embolization and antitubercular medications.

OUTCOME AND FOLLOW-UP

After 3 mo of mediastinal bronchial artery aneurysm embolization and initiation of antitubercular medication, patient become asymptomatic and no further episode of GI bleeding was encountered. Follow-up chest X-ray shows interval resolution of lung cavity.

DISCUSSION

Esophageal involvement in TB is extremely rare in otherwise healthy individuals. It mostly affects the mid-esophagus and is secondary to direct extension from the surrounding structures such as mediastinal lymph nodes, lungs, and vertebrae. The usual presenting symptoms are dysphagia or odynophagia[2]. There are several reports on esophageal TB that appeared as submucosal lesions mimicking a mass, ulcer, diverticulum, or only sinus opening[1].

Bronchial artery aneurysm is a rare condition with reported rate of < 1% in selective bronchial arteriogram[6]. It is can be classified according to location as mediastinal or intrapulmonary. Clinical presentation is variable depending on the size, location, concomitant disease and if it is ruptured or not. Although it usually detected incidentally on chest CT, the most frequent symptoms are hemoptysis, chest pain, and hemomediastinum[7]. Chest CT is greatly helpful in detecting TB manifestation in the chest and mediastinal lymph nodes as well as associated fistula formation[8]. CT angiography and conventional angiography are commonly used tools to diagnose mediastinal bronchial aneurysm[3].

Mediastinal bronchial artery aneurysms are fatal and even asymptomatic, requiring treatment regardless of the diameter. Endovascular embolization is the first line of treatment, and surgery is reserved as the last option for some patients with contraindication to endovascular therapy[3,5,7]. Anti-TB medication is the mainstay treatment of mediastinal TB. A large esophageal fistula secondary to TB could be treated by surgery or endoscopic clipping[2,8].

In this case report, although TB was suspected from the beginning, the presence of acute upper GI bleeding made it challenging to determine its source and treat it. Endoscopy and bronchoscopy – the examinations of choice for upper GI bleeding and suspected tracheoesophageal fistula – failed to identify the source. Chest CT with IV contrast and conventional angiography followed by coil embolization were of great value for diagnosis and treatment of such a rare condition. We believe that TB is a

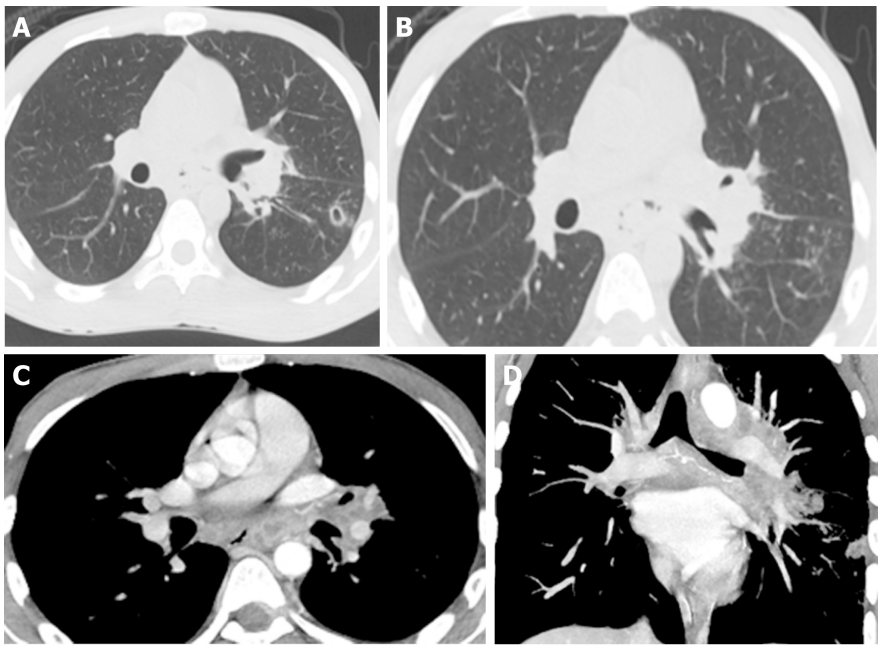

Figure 2 Computed tomography chest. A: Computed tomography lung window axial image shows left lower lobe lung cavitary lesion with multiple nodules in tree-in-bud configuration, a classical sign of lung tuberculosis; B: Chest computed tomography lung window axial image shows multiple mediastinal air pockets anterior to the esophagus, indicating an esophageal fistula; C: Computed tomography chest with IV contrast mediastinal window axial image shows multiple mediastinal necrotic lymph nodes and mediastinal air pocket anterior to esophagus indicating mediastinal tuberculosis and esophageal fistula; D: Computed tomography chest with IV contrast maximum intensity projection coronal reformatted image shows mediastinal bronchial artery aneurysm.

primary disease that involves the lung and then extends to the mediastinum and leads to esophageal fistula and mediastinal bronchial artery aneurysm formation. This rare presentation of acute upper GI bleeding is most likely due to mediastinal bronchial artery aneurysm that caused bleeding and drained into the esophagus by the esophagomediastinal fistula.

CONCLUSION

We successfully treated a patient who presented with acute upper GI bleeding due to tuberculous esophagomediastinal fistula and mediastinal bronchial artery aneurysm using transcatheter coil embolization.

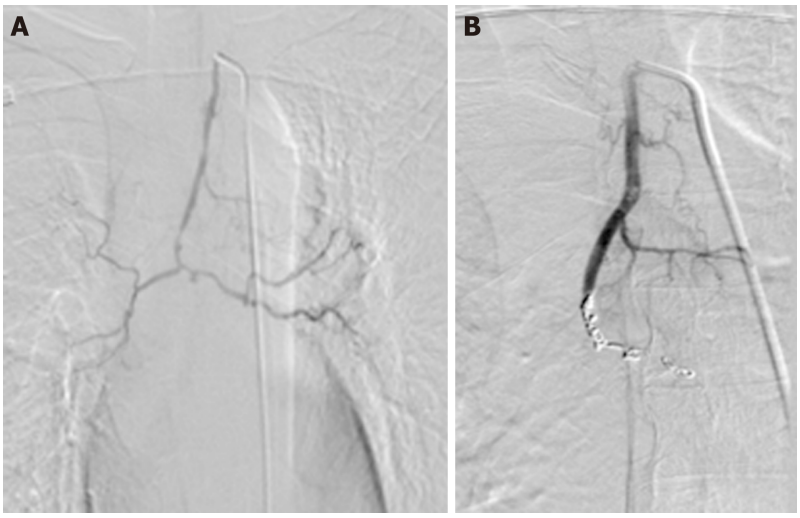

Figure 3 Conventional angiography. A: Selective angiogram of the bronchial artery showing 2 small mediastinal aneurysms; B: Post-coil-embolization angiogram shows complete occlusion of the artery and aneurysms.

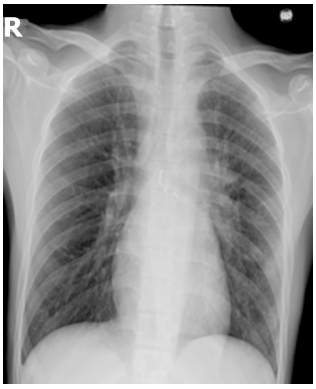

Figure 4 Follow-up chest X-ray shows resolution of cavitary lesion and no consolidation. Coils are seen in the mediastinum.

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