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EDITORIAL

Pharmacological cyclin dependent kinase inhibitors: Implications for colorectal cancer

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Abstract

Colorectal cancer accounts for a significant proportion of cancer deaths worldwide. The need to develop more chemotherapeutic agents to combat this disease is critical. Cyclin dependent kinases (CDKs), along with its binding partner cyclins, serve to control the growth of cells through the cell cycle. A new class of drugs, termed CDK inhibitors, has been studied in preclinical and now clinical trials. These inhibitors are believed to act as an anti-cancer drug by blocking CDKs to block the uncontrolled cellular proliferation that is hallmark of cancers like colorectal cancer. CDK article provides overview of the emerging drug class of CDK inhibitors and provides a list of ones that are currently in clinical trials.

Key words: Colorectal cancer; Cyclin; Cyclin dependent kinase inhibitor

Core tip: This article provides a brief overview of an emerging drug class, cyclin-dependent kinases inhibitors and their potential implications in colorectal cancer treatment.

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INTRODUCTION

In the United States, colorectal cancer (CRC) is among the three most common causes of death and accounts for approximately 8.4% of all cancer related deaths[1]. Colorectal cancer is primarily asymptomatic in earlier stages and therefore, often not diagnosed until the later stages of the disease. Currently, surgical

intervention with chemotherapy is the primary treatment for patient[2]. In metastatic disease, systemic chemotherapy is coupled with surgical resection of metastases, which are commonly seen in the liver[3,4]. A deeper understanding of the intricate molecular biology that mediates the pathogenesis of this disease may be beneficial in identifying new drug targets with the ultimate goal of improving survival time.

GOALS AND LIMITATIONS OF CURRENT THERAPEUTICS

Current therapeutic agents against cancerous cells aim to disrupt the rapid proliferation of cancerous cells; this is achieved either through directly targeting and killing cancerous cells or indirectly by slowing down cell growth. These drugs can be given systemically *via* oral medication or in a more targeted manner by direct injection into the blood[5]. Using drugs in combination has been proven to increase survival time and can prevent some drugs from developing resistance. Molecular pathways that mediate the processes of apoptosis, angiogenesis, invasion and the cell cycle are main targets of chemotherapy[6]. By understanding how molecular mechanisms regulate essential cellular processes, chemotherapeutic agents can be developed to combat the cancerous cells. The mammalian cell cycle, for example, is precisely regulated during periods of development and growth. This regulation is essential for proper cell differentiation and proliferation. Any loss of control over the events of the cell cycle can lead to unregulated growth and is associated with cancer development[7].

There have been several chemotherapeutic drugs that are FDA approved and are currently used in cancer treatment. Development of resistance to chemotherapeutic agents, difficulties in controlling metastatic disease and devastating side effects to drugs are only some of the limitations of the current arsenal of drugs[8,9]. The various limitations to the current drugs available that make the need to discover new drugs even greater. In this article, the authors aim to provide an update of search of cyclin-dependent kinases (CDK) inhibitors as an anti-cancer drug and provide information on new areas for therapy.

OVERVIEW OF CYCLINS AND CYCLIN DEPENDENT KINASES

Disruptions of the cell cycle have been well documented to be involved in the genesis and propagation of a variety of cancers, including colorectal cancer. The cell cycle is divided into two broad stages: mitosis and interphase. Mitosis is characterized as a period of division, whereas interphase is a period of metabolic growth[10]. The cell cycle itself is closely regulated by cyclins, a protein that activates CDK, a group of serine/threonine proteases. Cyclins serve as the regulatory unit that is vital for CDK activity; it is the interaction of cyclins with CDKs that helps mediate normal development and proliferation of mammalian cells[11]. Alterations in the function of any of the cell cycle regulator proteins such as cyclins and CDK are a hallmark of cancer development[12].

There have been numerous families of cyclins identified that are associated with specific stages of the cell cycle. Although different families of cyclins differ in primary amino acid sequence, they all share a common 100 amino acids sequence termed the cyclin box. This structure is responsible for binding to cyclin dependent kinases[13]. When a cyclin binding partner binds to its respective CDKs, it is now in the active form and can serve as a modulating signal that allows for progression through the cell cycle. Typically, these kinases are serine or threonine kinases that belong to a larger family of kinases that includes mitogen-activated kinases and glycogen synthase kinases[14]. There currently have been over nine CDK' s identified, with five of them being directly implicated with regulation of specific checkpoints in the cell cycle. Activated CDK's serve a regulatory role in the cell cycle and in transcription[15].

CDK INHIBITORS

It has been established in previous literature that deregulation in the function or mutation of the structure of CDK can result in disease processes[16,17]. Any changes in the levels of CDK interacting proteins can impact the phosphorylation of CDK[18]. For example, the loss of Cables, a CDK interacting protein, is linked to development of cancers including colon cancer[19]. The intricate and complex binding between cyclins and CDKs is difficult to mimic in vitro and success to fully elucidate the binding pattern has been limited[20]. In recent years, studies have been done to understand more about the roles of CDK inhibitors and if they can regulate uncontrolled cellular proliferation.

There are two families of CDK-inhibitors: INK4 and CIP/KIP class[21]. These two families differ in the particular cyclin families that they interact with. The inhibitor of CDK4 family, or INK4 consists of four individual proteins that selectively inhibit the D family of cyclins. The kinase inhibitor protein family or CIP/KIP is composed of three proteins that act to interact with other cyclin families. The inhibitor of CDK4 family, or INK4 consists of four individual proteins that selectively inhibit the D family of cyclins. The kinase inhibitor protein family or CIP/KIP is composed of three proteins that act to interact with other cyclin families[22]. From a mechanistic standpoint, it has been theorized that CDK-inhibitors can be used as an anti-cancer drug by blocking CDK's and therefore halting the uncontrolled cellular proliferation seen in cancer. Flavopiridol was the first CDK inhibitor ever tested in human clinical trials. This drug showed promise in preclinical cellular studies and was initially tested in patients with a

variety of carcinomas, including colon[23]. This drug was the first of it's kind to be tested in a clinical trial and set the stage for more investigation and further study of CDK inhibitors. Unfortunately, flavopiridol has had limited success individually and in combination with other medications as an anti-cancer drug. Since the testing of the first CDK inhibitor, flavopiridol, several other CDK inhibitors have been developed and tested. Inhibitors have been developed that target various stages of the cell cycle based on the specific cyclin-CDK blocked.

PHARMACOLOGICAL CDK INHIBITORS

There is great variability in the range of action of CDK inhibitors - some target specific points in the cell cycle while others are pan-CDK inhibitors that act much more broadly. This cell cycle attenuating effect documented in the literature of cellular CDK inhibitors implicate the use of pharmacological CDK inhibitors as an anti-cancer drug. There are several that have entered clinical testing. Here is a selected list of CDK–inhibitors that have entered or are in various stages of clinical trials[24].

CDK-INHIBITORS AND COLORECTAL CANCER

Based on preclinical research on colorectal cancers, there have been a few CDK alterations that have been strongly linked to the progression of the cancer. Understanding these cellular changes can serve as a potential indicator of prognosis and help scientists focus on the areas needed in drug development. The overexpression of CDK1 has been associated with increased risk of metastasis[4]. CDK2 overexpression has been seen in roughly 86% carcinomas[25]. Lastly, CDK4 overexpression is linked to a poor prognosis in colorectal cancer[26].

From the list of inhibitors currently in clinical trials, found in Table 1[23,27-41], there have been a few that have been studied more specifically in colorectal cancer. One that has shown particular promise is PD-0332991, from Pfizer, New York, NY. This is an inhibitor of CDK4 and CDK6. Studies done in human carcinoma cells have shown inhibited growth[42]. Additional studies done in mice with human colon carcinoma, Colo-205, have shown significant tumor regression[43]. This drug has shown promise as a clinically useful CDK-inhibitor that can potentially be used in colorectal cancers. Phase I clinical studies have been conducted that have shown positive results in patients with colorectal cancers[44,45].

LIMITATIONS TO CDK-INHIBITORS

A major difficulty in identifying CDK-inhibitors is finding one that has the potential to target a specific cyclin; a majority that have entered clinical trials either directly impact multiple cyclins/CDKs or have off target effects[46]. The poor selectivity between different CDKs results in a higher dose of CDK-inhibitor being used[47]. Lower doses of CDK-inhibitors used in trials have proved to be inefficient; yet, the proper management of dosage is critical to avoid reaching toxic doses. The challenges in finding a highly selective CDK-inhibitor has resulted in higher dosage that renders the side effects seen from CDK inhibitors in clinical trials become a limiting factor for their use. For example, flavopiridol has a dose-limiting toxicity of secretory diarrhea[48]. This inhibitor is a pan-CDK inhibitor and therefore requires a higher dose to elicit a cytotoxic effect. The high degree of homology amongst the group of CDKs, particularly in the ATP-binding site, has made it quite challenging to identify selective targets against particular CDKs[49]. Further work must be done to identify more selective molecular signatures of individual CDKs in preclinical studies to develop more targeted CDK-inhibitors.

Despite the promise that CDK-inhibitors hold, it is important to be cautious of any potential limitations. These compounds were identified over a decade ago and have been studied ever since. Although a few CDK-inhibitors have entered clinical trials, none have yet to show the promise of being a potent anti-cancer drug. CDK-inhibitors are a type of kinase inhibitor. A major concern that has been noted with the use of kinase inhibitors is the possibility of acquired drug resistance over the treatment regimen. For example, Imatinib, a well-established tyrosine kinase inhibitor, is known to cause an acquired resistance during the treatment course[50]. This resistance can be conferred through point mutations in the oncogene that result in the drug becoming less effective during the treatment regimen[51]. Currently, there have not been any reported incidents of CDK-inhibitors resulting in acquired resistance in the literature. However, it is important to note that there are highly conserved side chains present in CDKs that are specific targets of CDK-inhibitors[52]. Any point mutation or alteration of the unique molecular signature that CDK-inhibitors target can potentially confer resistance to the drug.

POTENTIAL ALTERNATIVES BESIDES CDK-INHIBITORS

There is a clear association between the role of cyclins and cyclin dependent kinases in regulation of the cell cycle. The research and studies of CDK-inhibitors have proved that much more work must be done. Instead of focusing on CDK-inhibitors, it would be wise to investigate other potential aspects of cyclins and CDKs that can be harnessed to develop therapeutics. We have chosen to discuss two other methods of potentially interrupting the cell cycle to create an anti-cancer drug: Cyclin-groove inhibitors and the

Table 1 Cyclin dependent kinases-inhibitors that have entered clinical trials

Drug name	Cyclin/CDK targeted	Manufacturer	Potential implications
Flavopiridol	Cyclin D and CDK-4/6 Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin A and CDK-1	Sanofi-Aventis, Bridgewater, NJ, United States	Renal cancer, Prostate cancer, colon cancer, non-Hodgkin's lymphoma[23]
PD-00332991	Cyclin D and CDK-4/6	Pfizer, New York, NY, United States	Breast cancer[25] Gastrointestinal tumors[26]
P276-00	Cyclin D and CDK-4/6 Cyclin E and CDK-2 Cyclin A and CDK-2	Piramal Life Sciences Limited, Mumbai, India	Mantle Cell Lymphoma[27]
PHA-848125	Cyclin D and CDK-4/6 Cyclin E and CDK-2 Cyclin A and CDK-2	Nerviano Medical Sciences, Nerviano, Italy	Metastatic solid tumors[27,28]
LY2835219	Cyclin D and CDK-4/6	Eli Lilly, Indianapolis, IN, United States	Lung cancers[29]
LEE011	Cyclin D and CDK-4/6	Novartis, Basel, Switzerland	Gastrointestinal cancers[30] Breast Cancer[31]
AZD5438	Cyclin D and CDK-4/6 Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin A and CDK-1	Astra Zeneca, London, England	Solid malignancies[32]
BAY 1000394	Cyclin D and CDK-4/6 Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Bayer, Barmen, Germany	Small cell lung cancer[33]
P1446A-05	Cyclin D and CDK-4/6	Piramal Life Sciences Limited, Mumbai, India	Advanced malignancies[34]
SNS-032	Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Sunesis Pharmaceutical, South San Francisco, CA, United States	Multiple myeloma, chronic lymphocytic leukemia[35]
Bryostatin-1	Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Ellisville, MO, United States	Metastatic renal cell carcinoma, soft tissue sarcoma[36]
Roscovitine	Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Cyclacel Pharmaceuticals, Short Hills, NJ, United States	Advanced malignancies[37]
Dinaciclib	Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Merck, Whitehouse Station, NJ, United States	Breast Cancer[38]
UCN-01	Cyclin E and CDK-2 Cyclin A and CDK-2 Cyclin B and CDK-1	Sigma-Aldrich. St. Louis, MO, United States	Breast Cancer[39]

CDK: Cyclin dependent kinase.

disruption of the cyclin-CDK interaction through peptides.

The cyclin binding groove is utilized in substrate recruitment. Theoretically, being able to place a cyclin groove inhibitor (CGI) peptide in this pocket would prevent cyclin from binding to CDKs[53]. The CGI peptide could potentially be used as an anti-cancer drug that acts by stopping the characteristic uncontrolled proliferation seen in cancer. Preclinical studies using cell permeable CGI peptides have been done in cellular models and *in vivo* using a mouse tumor model. These studies have shown promising results of blocking CDK activity[54]. The process of identifying and understanding the intricate protein structure and binding has been a hurdle in discovery of a CGI peptide suitable for clinical trial[55]. However, this is an avenue of research that should be investigated further and actively pursued.

An essential and critical step required for CDK control over the cell cycle is the disruption of the cyclin-CDK interaction. Studies have identified a peptide termed NBI1 that acts as a non-competitive inhibitor with certain cyclins[56]. Other preclinical studies in cell models have garnered more information about this novel peptide[57]. Further studies must be done in order to fully understand the capabilities of this peptide and see if there is a potential for clinical application. There are still many aspects of cyclin-CDK interactions that are not fully understood, so there may be other ways besides the use of CDK-inhibitors to disrupt uncontrolled cellular proliferation.

CONCLUSION

The discovery of cyclins and their enzyme effectors, the cyclin dependent kinases, has been monumental to deepening the understanding of the intricate and complex regulatory mechanisms of cell cycle. The mission to develop CDK inhibitors as a potent anti-cancer drug has been excellent in theory but limited in clinical results. Combination therapies with CDK-inhibitors and other cancer drug types have yielded better results but continued work must be done to increase the specificity and effectiveness of these

treatments. Further work must be done in both preclinical studies and clinical studies to identify and understand the complex molecular mechanisms that regulate the cell cycle.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

Diagnostic imaging and radiation exposure in inflammatory bowel disease

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Abstract

Diagnostic imaging plays a key role in the diagnosis and management of inflammatory bowel disease (IBD). However due to the relapsing nature of IBD, there is growing concern that IBD patients may be exposed to potentially harmful cumulative levels of ionising radiation in their lifetime, increasing malignant potential in a population already at risk. In this review we explore the proportion of IBD patients exposed to high cumulative radiation doses, the risk factors associated with higher radiation exposures, and we compare conventional diagnostic imaging with newer radiation-free imaging techniques used in the evaluation of patients with IBD. While computed tomography (CT) performs well as an imaging modality for IBD, the effective radiation dose is considerably higher than other abdominal imaging modalities. It is increasingly recognised that CT imaging remains responsible for the majority of diagnostic medical radiation to which IBD patients are exposed. Magnetic resonance imaging (MRI) and small intestine contrast enhanced ultrasonography (SICUS) have now emerged as suitable radiation-free alternatives to CT imaging, with comparable diagnostic accuracy. The routine use of MRI and SICUS for the clinical evaluation of patients with known or suspected small bowel Crohn's disease is to be encouraged wherever possible. More provision is needed for out-of-hours radiation-free imaging modalities to reduce the need for CT.

Key words: Diagnostic medical radiation; Inflammatory bowel disease; Small bowel follow-through; Computerised tomography; Nuclear medicine; Magnetic resonance enterography; Small intestine contrast-enhanced ultrasonography

Core tip: Due to the chronic and relapsing nature of inflammatory bowel disease (IBD), patients are at risk of exposure to potentially harmful cumulative radiation

doses in their lifetime. Computed tomography (CT) imaging remains responsible for the majority of this radiation exposure. As well as new reduced radiation CT imaging techniques, radiation-free alternatives magnetic resonance imaging and small intestine contrast enhanced ultrasonography have emerged, offering comparable diagnostic accuracy. In this review we explore the proportion of IBD patients exposed to high cumulative radiation doses, the factors associated with higher radiation exposures, and we compare conventional imaging with newer radiation-free imaging techniques for the evaluation of patients with IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD), consisting of ulcerative colitis (UC) and Crohn's disease (CD), is a chronic relapsing-remitting inflammatory disorder of the gastrointestinal tract. The prevalence of IBD is increasing worldwide, with 2.2 million and 1.4 million people affected in Europe and United States respectively[1].

Diagnostic imaging is required to aid the diagnosis of IBD, assess disease extent and severity, detect complications including extra-intestinal manifestations, and monitor response to treatment. Due to the relapsing nature of IBD, multiple imaging studies are often required. Despite this, in clinical practice cumulative exposure to radiation is not routinely monitored.

Patients with IBD have an increased lifetime risk of developing colorectal and small intestinal cancers, irrespective of diagnostic radiation exposure[2,3]. There is growing concern that repeated X-ray based imaging may additionally expose this typically young cohort of patients to harmful cumulative levels of ionising radiation, further increasing their lifetime cancer risk.

In this article we review the proportion of IBD patients exposed to potentially harmful cumulative radiation doses and the risk factors associated with higher radiation exposures. We explore and compare conventional diagnostic imaging and newer radiation-free imaging techniques for the evaluation of patients with IBD.

RADIATION EXPOSURE AND CANCER RISK

Extensive study of the atomic bomb survivors from Hiroshima and Nagasaki has formed the basis for quantitative estimates of radiation-induced cancer risk. In this large cohort of survivors, the rates of solid cancer deaths were positively associated with higher radiation doses and younger age of exposure[4,5]. In a 2012 study of the atomic bomb survivors, the relative risk of solid cancers increased by 29% per decade decrease in the initial age of radiation exposure[5]. Younger people appear to be inherently more radiosensitive, and have more remaining life-years during which a cancer may develop[4].

It is estimated that diagnostic medical radiation (DMR) exposure may be responsible for up to 2% of cancers worldwide[6]. Younger patients and females appear to have the greatest radiation-induced cancer risk[7]. Epidemiological data suggests that ionising radiation levels as low as 50 millisieverts (mSv) have been implicated in the development of solid tumours[8]. Potentially harmful radiation exposure is, therefore, commonly defined as cumulative effective dose (CED) > 50 mSv; the equivalent of five computed tomography (CT) abdominal-pelvis scans. A reference table comparing radiation exposure doses of common diagnostic gastrointestinal imaging techniques is included in Table 1[9,10].

Table 1 Radiation doses from gastrointestinal imaging studies in comparison to background radiation, condensed from RadiologyInfo.org[9] and Mettler *et al*[10]

Imaging procedure	Average effective dose (mSv)	Time period for equivalent effective dose from natural background radiation[1]
Multiphase CT abdomen and pelvis	31	10.3 yr
PET/CT	25	8.3 yr
CT Abdomen and Pelvis	10	3.3 yr
CT Colonography	10	3.3 yr
CT Abdomen	8	2.7 yr
Barium Enema	8	2.7 yr
Small bowel follow-through	5	1.7 yr
X-ray abdomen	0.7	2.8 mo

[1]Based on the assumption of an average effective dose of 3 mSv per year from natural background radiation. CT: Computed tomography; PET: Positron emission tomography.

CUMULATIVE RADIATION EXPOSURE IN IBD PATIENTS

Several published studies have attempted to quantify the proportion of IBD patients exposed to potentially harmful cumulative levels of ionising radiation (summarised in Table 2). Desmond *et al*[11] first evaluated DMR exposure in 354 patients with CD in a single tertiary centre in Ireland. CT imaging accounted for 77.2% of the total DMR exposure. The mean CED was 36.1 mSv and exceeded 75 mSv in 15.5% of patients. More recently, a meta-analysis by Chatu *et*

Table 2 Quantification of the cumulative effective dose of diagnostic radiation received by IBD patients, and factors associated with high cumulative radiation exposure (cumulative effective dose > 50 mSv); adapted from Chatu *et al*[12] with permission

Study	Number of patients (*n*)	Country	Design	Patient population	Outcome CED ⩾ 50 mSv	Mean/Median CED (mSv)	Factors associated with high radiation exposure
Newnham *et al*[59], 2007	100 (62 CD, 37 UC, 1 indeterminate colitis)	Australia	Retrospective study, single tertiary centre, patients recruited consecutively from clinic	Adult (16-84 yr)	11/100 (11%) 9 CD, 2 UC	Median CED 10 mSv	Assessed: age, gender, disease, disease duration, previous surgery, immunomodulator use, referral source Significant: none
Desmond *et al*[11], 2008	354 CD	Ireland	Retrospective study, single tertiary centre, patients recruited from IBD database July 1992-June 2007	Adult and paediatric (8.6-78.3 yr)	CED ⩾ 75 mSv in 55/354 patients (15.5%)	Mean CED 36.1 mSv	Assessed: age, gender, smoking, FH, disease distribution, disease behaviour, medication, surgical history Significant: age < 17 at diagnosis, upper GI tract disease, penetrating disease, requirement for Ⅳ steroids, infliximab use, multiple surgeries
Peloquin *et al*[18], 2008	215 (103 CD, 112 UC)	United States	Retrospective study, population based inception cohort diagnosed between 1991 to 2001 from Olmsted County	Adult and paediatric (1.2-91.4 yr)	N/A	Median CED CD: 26.6 mSv UC: 10.5 mSv	N/A
Levi *et al*[60], 2009	324 (199 CD, 125 UC)	Israel	Retrospective study, single tertiary centre, patients diagnosed Jan 1999-Dec 2006, recruited from IBD database	Adult and paediatric ⩽ 17 yr (18) > 18 yr (306)	23/324 (7.1%)	Mean CED CD: 21.1mSv UC: 15.1mSv	Assessed: age, surgery, diagnosis, medical therapy, disease duration, gender Significant: CD, surgery, prednisolone use, disease duration, first year of disease, age
Palmer *et al*[61], 2009	1593 (965 CD, 628 UC)	United States	Retrospective study, population based cohort recruited from insurance claims database Jan 2003-December 2004	Paediatric (2-18 yr)	N/A (34% CD, 23% UC exposed to moderate radiation - at least 1 CT or 3 fluoroscopic procedures)	N/A	Assessed: age, gender, region, hospitalisation, surgery, ED encounter, medication Significant: hospitalisation, inpatient GI surgery, ED encounter, use of steroids
Kroeker *et al*[62], 2011	553 (371 CD, 182 UC)	Canada	Retrospective study, single tertiary centre, patients diagnosed 2003-2008, recruited from IBD database	Adult and paediatric (15-84 yr)	28/553 (5%) 27 CD, 1 UC	Mean CED CD: 14.3 mSv UC: 5.9 mSv	Assessed: age at diagnosis, gender, disease distribution, previous surgery Significant: previous surgery
Fuchs *et al*[63], 2011	257 (171 CD, 86 UC)	United States	Retrospective study single tertiary centre, patients reviewed Jan-May 2008	Paediatric (< 18 yr)	15/257 (5.8%) 14 CD, 1UC	Mean CED CD: 20.5 mSv UC: 11.7 mSv	Assessed in CD cohort: gender, disease behaviour, previous surgery, disease duration, elevated platelet count at diagnosis Significant: previous surgery, elevated platelet count at diagnosis
Sauer *et al*[64], 2011	117 (86 CD, 31 UC)	United States	Retrospective study, single tertiary centre, patients diagnosed 2002-2008	Paediatric (2-18 yr)	6/117 (5%) 6 CD	Median CED CD: 15.6 mSv UC: 7.2 mSv	N/A

Huang *et al*[65], 2011	105 (61 CD, 32 UC, 12 indeterminate colitis)	United States	Single tertiary paediatric centre, patients identified from medical records	Paediatric cohort (11 mo-18 yr)	6/105 (6%)	Mean CED 15 mSv	Assessed: surgery, disease type, disease location, racioethnic background, anti TNF agents, use of immunomodulators, hospital admissions, age at diagnosis Significant: CD, small bowel involvement, black ethnicity, number of hospital admissions, previous surgery, anti TNF alpha use
Butcher *et al*[17], 2012	280	United Kingdom	Retrospective study, single tertiary centre, consecutive patients attending IBD clinic	Adult cohort	6.3% CD	Mean CED 10.17 mSv Median CED 4.12 mSv	Significant: smoking status, disease duration, previous surgery
Jung *et al*[15], 2013	2199 (777 CD, 1422 UC)	South Korea	Retrospective study, multicentre conducted at 13 university hospitals in South Korea, patients diagnosed July 1987-Jan 2012 included	Adult cohort (Mean age: CD 29.2 yr; UC 42.2 yr)	34.7% CD, 8.4% UC	Mean CED CD: 53.6 mSv UC: 16.4 mSv	Assessed: gender, age at diagnosis, disease duration, disease extent, surgery, hospitalisation, 5-ASA use, steroids, immunomodulator use Significant: For CD - longer disease duration, ileocolonic disease, upper GI tract involvement, surgery, hospitalisation, steroids For UC - surgery, hospitalisation, infliximab use
Chatu *et al*[14], 2013	415 (217 CD, 198 UC)	United Kingdom	Retrospective study, single tertiary centre, patients consecutively recruited from clinic Jan 2011- June 2011	Adult cohort (Mean age: CD 30.8 yr; UC 36.9 yr)	32/415 (8%) 29 CD, 3 UC	Median CED CD: 7.2 mSv UC: 2.8 mSv	Assessed: gender, age at diagnosis, disease type, steroid use within 3 mo diagnosis, use of immunomodulators or biologics, extraintestinal features, IBD related surgery Significant: males, IBD related surgery
Estay *et al*[13], 2015	325 (82 CD, 243 UC)	Chile	Retrospective study, patients recruited from IBD Registry 2011-2013	Adult cohort (16-86 yr)	22/325 (6.8%): CD 16 (19.5%); UC 6 (2.5%)	Mean CED 11.97 mSv CD: 29.9 mSv UC: 5.92 mSv	Assessed in CD cohort only: age at diagnosis, disease duration, disease location, disease behaviour, perianal disease, surgery, hospitalisation, medications Significant: longer disease duration, ileal involvement, stricturing disease, treatment with steroids and biological agents, CD related hospitalisation or surgery

UC: Ulcerative colitis; CD: Crohn's disease; IBD: Inflammatory bowel disease; CED: Cumulative effective dose; mSv: Millisieverts of radiation; ED: Emergency department; FH: Family history.

al[12], evaluated six studies including a total of 1704 IBD patients. It reported a pooled estimate of 8.4% of IBD patients receiving high dose radiation exposure (CED > 50 mSv). More patients with CD (11.1%) were exposed to high cumulative radiation doses (CED > 50 mSv) than patients with UC (2%)[12].

Similar trends have been found in studies following this meta-analysis. A 2015 retrospective review of 325 IBD patients in Chile, reported 19.5% of patients with CD and 2.4% of patients with UC, to be exposed to CED > 50 mSv[13]. A recent United Kingdom retrospective study of 415 patients with IBD referred from primary care, reported a median total CED of 7.2 mSv in CD patients and 2.8 mSv in UC patients, with 8% of IBD patients overall exposed to CED > 50 mSv. Kaplan Meier analysis projected a probability

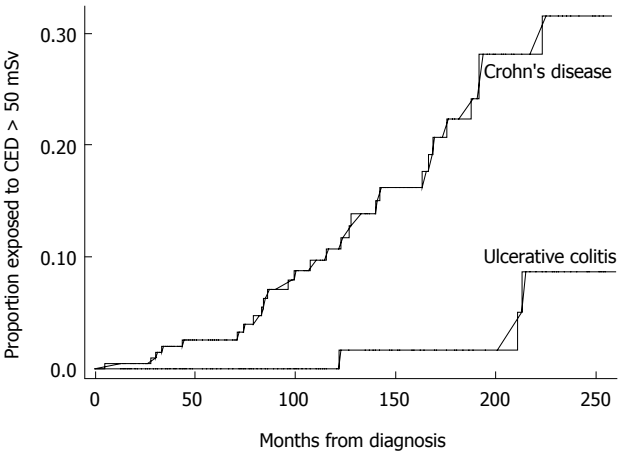

Figure 1 Kaplan Meier analysis showing the cumulative probability of being exposed to cumulative effective dose > 50 mSv from diagnosis according to inflammatory bowel disease type (Chatu *et al*[14], 2013).

of exposure to CED > 50 mSv of 6% and 14% at 10 years and 15 years from IBD diagnosis respectively (Figure 1)[14].

Concerningly, a retrospective study of IBD patients in South Korea, conducted across 13 university hospitals, reported even higher proportions of patients exposed to potentially harmful radiation levels. Thirty-four point seven percent of patients with CD and 8.4% of patients with UC were exposed to CED > 50 mSv[15]. CT imaging accounted for the vast majority of this radiation exposure (81.6% of the total CED in CD *vs* 71.2% in UC)[15], indicating that overuse of CT imaging remains a concern worldwide, and may reflect limited availability or lack of awareness of preferable imaging modalities.

Despite the high proportion of IBD patients exposed to high radiation doses, cumulative radiation exposures are not routinely recorded in clinical practice. The creation of IBD radiation diaries has been proposed to log total radiation exposures[16], and improve recognition among physicians where a patient has previously been exposed to ionising radiation.

FACTORS ASSOCIATED WITH INCREASED RADIATION EXPOSURE IN IBD PATIENTS

Risk factors for high radiation exposure in IBD patients have been widely studied[11-15,17]. In a cohort of 354 adult and paediatric patients with CD, Desmond *et al*[11] identified that patients diagnosed under the age of 17, patients with upper gastrointestinal (GI) disease, penetrating disease, multiple surgeries, or those that required intravenous steroids or infliximab, were at greater risk of receiving high cumulative radiation exposure. Following this, a 2012 meta-analysis of five studies evaluating risk factors in 2627 IBD patients, found a significant association with only previous IBD related surgery and corticosteroid use. The pooled adjusted odds ratios were 5.4 and 2.4, respectively[12].

Across studies, patients with CD consistently appear to receive higher cumulative radiation exposures than patients with UC, possibly due to a greater likelihood of extraluminal complications commonly examined by CT. After adjusting for time since symptom onset, a retrospective study by Peloquin *et al*[18] (2008) found patients with CD to be exposed to 2.46 times more diagnostic radiation than patients with UC (median CED 26.6 mSv in CD *vs* 10.5 mSv in UC).

A summary of outcomes from studies investigating predictive factors for high radiation exposure in IBD patients is provided in Table 2. While there are discrepancies regarding the significance of some associations, the majority of the risk factors described are surrogate markers of disease activity and severity. It is therefore apparent that patients with more severe disease, who are more likely to receive corticosteroids and require surgery, undergo more diagnostic imaging including greater use of CT imaging, to guide further management.

DIAGNOSTIC IMAGING MODALITIES IN IBD

Small bowel follow-through

A 2011 survey revealed small bowel follow-through (SBFT) to be the most frequently performed investigation in the United Kingdom for the assessment of small bowel CD[19]. CT was predominantly performed for suspected extra-luminal complications or obstruction[19]. SBFT and small bowel enteroclysis (SBE) have, for many years, been the routine first-line imaging modalities to evaluate small bowel involvement in patients with suspected or confirmed CD. Both SBFT and SBE have similar sensitivities (85%-95%) and specificities (89%-94%) for detecting radiological features of CD[20]. SBFT is usually preferred for patient tolerance, since nasal or oral intubation is not required. However, these techniques both employ ionising radiation and appear to have lower diagnostic accuracy compared to newer cross-sectional imaging modalities[21,22].

In a 2005 United States study, SBFT had a lower diagnostic yield for mild to moderate CD compared to CT enterography, video capsule endoscopy and ileoscopy[21]. A 2009 Korean study of 30 patients with CD, found a significantly lower sensitivity of SBFT for the detection of extra-enteric complications ($P < 0.01$), although no significant difference in the detection of active terminal ileitis, compared to CT and magnetic resonance enterography (MRE)[22]. Barium based studies may still have a role to play in the evaluation of small bowel CD, but are increasingly being replaced by alternative imaging modalities such as CT, MRE and small bowel ultrasound.

CT

In the United States, CT has largely superseded

SBFT as the preferred first-line imaging modality for CD. Between 2002 and 2007, there was a reported 840% increase in the use of CT enterography in IBD patients in Minnesota, United States[6]. Similarly, a 310% increase in use of abdominal CT imaging was reported in a United Kingdom study of IBD patients between 1990 and 2010[14]. CT imaging offers the advantages of widespread availability, rapid acquisition of images, high sensitivity and specificity for the detection of intramural and extra-intestinal disease, as well as being well tolerated by patients[4]. The effective radiation dose is, however, considerably higher than other abdominal imaging modalities (Table 1)[9,10]. The United States National Research Council estimates that one out of every 1000 patients undergoing a 10 mSv CT scan will develop a radiation-induced cancer in their lifetime[23].

Conventional CT abdominal-pelvis imaging is typically used for the detection of extra-intestinal complications of IBD, such as abscesses, fistula, bowel obstruction or perforation. It may have a limited role in the assessment of colonic disease activity. A small study by Patel *et al*[24] of 23 patients with UC (2012), identified positive correlation of contrast-enhanced CT features (bowel wall thickening, mucosal hyper-enhancement and mural stratification), compared with clinical assessment ($P < 0.05$) and colonoscopy ($P < 0.0001$) in evaluating UC disease severity. However, only increasing bowel wall thickness on CT correlated with histological disease severity[24].

Conventional CT is limited in its assessment of small bowel inflammation due to artefact produced from collapsed bowel loops. CT enterography (CTE) is a newer imaging technique, combining high resolution CT scanning with multiplanar reconstructions after administration of an oral and parenteral contrast which acts to promote bowel loop distension. This improves visualisation of the small bowel mucosa, enabling more accurate assessment of small bowel disease activity[25]. High correlation has been shown between quantitative measures of bowel wall thickness and terminal ileal mural attenuation at CTE compared with ileocolonoscopy and histological analysis in active CD[26]. Furthermore, CTE may be a useful adjunct to ileocolonoscopy. In a 2012 study of 153 patients with CD in the United States, CTE detected active small bowel disease in 36 of the 67 patients (54%) with normal ileoscopy appearances. The negative ileoscopy results were largely due to disease "skipping" of the terminal ileum, or confinement to intramural or mesenteric distal ileum. CTE also detected extra-colonic CD in 26% of patients[27].

Data for the benefit of CTE in assessing colonic disease is limited. A small study analysing CTE in 35 patients with inflammatory colitis, identified a sensitivity of 93% and specificity of 91% for the detection of moderate to severe disease in well-distended colons. However, there was a tendency for CTE to underestimate the full extent and severity of colonic disease[28].

CT colonography (CTC) is an emerging imaging technique developed for colonic evaluation. While colonoscopy remains standard practice for the assessment of colonic disease, CTC may offer advantages where colonoscopy is incomplete or contra-indicated. The majority of data comparing CTC and colonoscopy has been obtained from studies detecting colorectal cancer[25]. Only a few studies have investigated the efficacy of CTC in IBD, hence its role is not clearly defined. A small German prospective study of 21 IBD patients suggested sensitivities of 63.6% and 100% for the identification of acute and chronic IBD by CTC, with a specificity of 75% and 100% respectively[29]. CTC requires full bowel preparation, as well as air or carbon dioxide insufflation for colonic distension, and therefore is not always well tolerated. There have been reported cases of CTC-induced bowel perforation as well. Although the perforation rate is low, at around 0.04%, CTC is generally avoided in the acute phase of IBD[30].

Unfortunately while CT performs very well as an imaging modality there is an emerging recognition that it is responsible for the majority of the total radiation dose to which IBD patients are exposed[11-13,15]. Indeed in a recent study from Chile, abdominal-pelvic CT and CT enteroclysis accounted for 93.6% of the total CED exposure[13]. Excessive use of CT imaging in IBD patients presenting to the emergency department (ED) has also raised concern. In a study from the United States, no significant findings were observed in 32.8% of CT imaging studies carried out in IBD patients in the ED[31]. Preliminary algorithms to avoid inappropriate use of CT imaging in IBD patients presenting to the ED have been proposed and require validation[31,32].

Reduced radiation dose CT

Due to concerns regarding high radiation exposure from CT imaging, recent developments in technology have paved the way for strategies to reduce the radiation dose associated with CT imaging, without compromising diagnostic imaging quality. These techniques include tube current (mA) modulation, lowering tube potential modulation (kV), and minimising the number of dynamic CT phases[33]. Multiphase CT abdomen and pelvis imaging exposes a patient to around 31 mSv, equivalent to over three times the radiation dose of standard CT abdominal-pelvis imaging[9]. Single-phase CTE is in most cases believed to be sufficient to evaluate small bowel CD[33]. Reduced radiation CT techniques may help to lessen cumulative radiation exposures and bridge the gap in situations where radiation-free imaging is not widely available.

Nuclear medicine imaging

Technetium-99-m hexamethyl-propyleneamine oxime (99mTc-HMPAO) labeled white blood cell scintigraphy is an imaging technique that employs radioactive isotopes

to detect active inflammation[34]. It may be used in IBD to assess disease activity, but due to limited availability and high cost, it is not routinely performed. 99mTc-HMPAO white cell scintigraphy can visualise the entire GI tract and emits a lower radiation dose than CT (2-4 mSv)[35]. Reported uses include evaluating responses to treatment and differentiating between disease relapse and fibrotic tissue post surgery[36]. It also has a role in assessing disease extent in acute severe colitis, where colonoscopy is usually contra-indicated. A United Kingdom study by Subramanian *et al*[37] of 135 patients with UC, noted substantial correlation (k = 0.7) between 99mTc-HMPAO white cell scintigraphy and histological assessment of the proximal extent of disease involvement in patients with UC. Scintigraphy performed better than colonoscopy (P = 0.02) in assessing patients with more extensive colitis, while colonoscopy predicted disease extent more accurately in patients with limited colitis (P = 0.002)[37].

Positron emission tomography (PET) is a non-invasive nuclear imaging technique that provides three dimensional, quantitative imaging. It is primarily used for tumour staging, though preliminary data has shown it may have some value in the diagnosis of IBD[38,39]. PET imaging is expensive and its availability is limited to certain centres. Data on the potential role of Fluorine-18-Fluorodeoxyglucose/PET (^{18}F-FDG/PET) and PET/CT in IBD is limited and requires further review. Routine use of PET/CT for IBD assessment is unlikely due to the high doses of radiation involved (Table 1)[9,10].

RADIATION-FREE DIAGNOSTIC IMAGING MODALITIES

In view of the concerns over cumulative radiation exposure in IBD patients, alternative radiation-free imaging strategies have emerged as a focus of interest, and are increasingly being favoured in clinical practice. Studies comparing the diagnostic accuracies for radiation-free imaging *vs* conventional imaging modalities in small bowel CD are summarised in Table 3.

A 2008 meta-analysis by Horsthuis *et al*[40] comparing magnetic resonance imaging (MRI), ultrasonography (US), scintigraphy and CT across 33 studies, showed high per-patient sensitivity for the diagnosis of IBD with no significant differences between imaging modalities. Mean sensitivity estimates were 93%, 90%, 88% and 84% for MRI, US, white cell scintigraphy and CT respectively. Per-patient specificity was also high and comparable across imaging modalities: 93%, 96%, 85% and 95% for MRI, US, scintigraphy and CT respectively. The only significant difference was a lower specificity for scintigraphy compared to US (P = 0.009)[40]. Mean per-bowel-segment sensitivity estimates were lower across all imaging modalities (70%, 74%, 77% and 68% for MRI, US, scintigraphy and CT respectively). Per-bowel-segment analysis showed CT to be significantly less sensitive and specific compared to MRI (P = 0.037) and scintigraphy (P = 0.006)[40]. More recently, a 2011 systematic review by Panés *et al*[41] also compared US, MRI and CT for the assessment of disease location and extension in CD. Overall, US had superior diagnostic accuracy for the detection of disease localised to the terminal ileum and colon, while MRI performed better than US for the detection of CD lesions in the jejunum and proximal ileum. CT and MRI demonstrated similar diagnostic accuracy for the assessment of CD extension and activity[41].

MRE

MRE is a non-invasive technique used to obtain cross-sectional imaging of the small bowel without exposure to diagnostic medical radiation. MRE provides superior soft tissue contrast resolution compared to CTE, allowing detailed visualisation of inflammatory and fibrotic bowel wall[42]. A 2011 Italian prospective study by Fiorino *et al*[43] compared MRE and CTE in 44 patients with ileocolonic CD. They found comparable accuracy between MRE and CTE in localising CD, assessing bowel wall thickening, bowel wall enhancement and enteroenteric fistula. However, MRE was superior to CTE in detecting strictures (P = 0.04) and ileal wall enhancement (P = 0.02). A 2014 meta-analysis by Qiu *et al*[44] of 290 CD patients across six studies, found no significant difference between the diagnostic accuracy of MRE and CTE in detecting active small bowel CD and its complications including fistula, stenosis and abscess formation.

Given its proven diagnostic accuracy, updated guidelines by the European Crohn's and Colitis Organisation and the European Society of Gastrointestinal and Abdominal Radiology, advocate increased routine usage of MRI for the assessment of small bowel CD, to reduce radiation exposure in this cohort of patients[45].

Recently, diagnostic indices from MRE have been developed to attempt to quantify disease severity. The magnetic resonance index of activity score has demonstrated a significant correlation with the CD endoscopic index of severity[42]. In perianal CD, MRI remains the preferred imaging modality, permitting accurate diagnosis and staging of perianal fistula[42]. Drawbacks of MR imaging, however, include higher procedure costs, lengthy acquisition times and limited availability, particularly out of routine working hours.

The efficacy of MR Colonography (MRC) for the evaluation of colonic disease activity in IBD is less well defined. A 2005 study comparing MRC using contrast gadolinium enemas, to standard colonoscopy in 22 patients with suspected or known IBD revealed disappointing results, with a per-segment sensitivity of 58.8% and 31.6% for identifying colonic inflammation in UC and CD respectively[46]. Other studies have

Table 3 Comparison of diagnostic accuracies of radiation-free imaging (ultrasonography/magnetic resonance imaging) *vs* conventional ionising radiation imaging for the evaluation of small bowel Crohn's disease

Study	Country	Number of patients (*n*)	Design	Imaging compared	Study findings
Low *et al*[66], 2000	United States	26 CD	Prospective study, single centre	Contrast enhanced MR with single phase CT using findings from surgery, barium studies, endoscopy and histology as reference standard	Side-by-side comparison: MR imaging superior than helical CT in depiction of normal bowel wall, mural thickening or enhancement and overall GI tract evaluation MR images showed 55 (85%) and 52 (80%) of 65 abnormal bowel segments for the two observers, compared with helical CT which showed 39 (60%) and 43 (65%) of bowel segments affected by CD ($P < 0.001$, $P < 0.05$)
Maconi *et al*[67], 2003	Italy	128 CD	Prospective study, consecutive CD patients who underwent surgery immediately after diagnostic work-up	US, barium studies, CT to detect internal fistulae and intra-abdominal abscesses compared to intraoperative findings	Detecting internal fistula: comparable diagnostic accuracy of US (85.2%) and barium X-ray (84.8%) studies Sensitivity US (71.4%), X-ray (69.6%), Specificity US (95.8%), X-ray (95.8%) Detection of abscesses: US (90.9%), CT (86.4%) Overall diagnostic accuracy higher with CT than US (91.8% *vs* 86.9%) due to false positives with US
Parente *et al*[49], 2004	Italy	102 CD	Prospective study, consecutive patients with proven CD by BE and ileocolonoscopy enrolled from IBD clinic Dec 2002-July 2003 Adult cohort (⩾ 18 yr)	Conventional US *vs* oral contrast enhanced US, compared to BE and ileocolonoscopy as gold standard	Per segment analysis: Superior diagnostic accuracy of contrast US in detecting small bowel CD. Sensitivity: conventional US 91.4%, contrast US 96.1% Good correlation of disease extent measurements with BE: US ($r = 0.83$), contrast US ($r = 0.94$) Higher sensitivity and specificity with contrast US in detecting ⩾ 1 small bowel strictures: Sensitivity: US (74%), contrast US (88.8%) Specificity: US (93.3%), contrast Us (97.3%) US and contrast US more accurate in detecting internal fistulas than BE, but no significant difference in diagnostic accuracy between US and contrast US. US (80%), contrast US (86%), BE (67%) Significantly improved interobserver variability between sonographers with contrast US for detecting bowel wall thickness and disease location
Calabrese *et al*[55], 2005	Italy	28 CD	Prospective study, consecutive patients recruited from IBD clinic Adult cohort (age range 21-60 yr)	SICUS (performed by a sonologist of 1 yr experience) *vs* TUS (performed by an experienced sonologist of 10 yr experience), compared to SBE as gold standard	Sensitivity for detection of small bowel lesions: 96% TUS, 100% SICUS Greater correlation of extension of lesions between SICUS and SBE ($r = 0.88$) *vs* TUS and SBE ($r = 0.64$) Sensitivity for detection of ⩾ 1 stricture: 76% TUS, 94% SICUS Sensitivity and specificity for assessing prestenotic dilatation: 50% and 100% for TUS, *vs* 100% and 90% for SICUS
Horsthuis *et al*[40], 2007	Amsterdam	1735 (sample size 15-440)	Meta-analysis of 33 prospective studies published between Jan 1993- Feb 2006 Adult and paediatric cohort (age range 2-86 yr)	US, MRI, scintigraphy, CT US evaluated in 11 studies, MRI in 11, scintigraphy in 9 and CT in 7 studies	Per-patient analysis: Significantly lower specificity for scintigraphy *vs* US. No significant difference between mean sensitivities for diagnosis of IBD Sensitivities: 89.7% US, 93% MRI, 87.8% scintigraphy, 84.3% CT Specificities: 95.6% US, 92.8% MR, 84.5% scintigraphy, 95.1% CT Per bowel segment analysis: Significantly lower sensitivity and specificity for CT compared to scintigraphy and MRI. Sensitivities: 73.5% US, 70.4% MRI, 77.3% scintigraphy, 67.4% CT. Specificities: 92.9% US, 94% MRI, 90.3% scintigraphy, 90.2% CT

Lee *et al*[22], 2009	South Korea	30 CD	Prospective study, single centre, consecutive patients with known or suspected CD enrolled Adult cohort (age range 18-44 yr)	MRE, CT, SBFT for detection of active small bowel inflammation and extra enteric complications with ileocolonoscopy as reference standard	No significant difference between CTE, MRE and SBFT for the detection of active terminal ileitis. Sensitivity CTE (89%), MRE (83%), SBFT (67%-72%) Significantly higher sensitivity for MRE (100%) and CTE (100%) compared to SBFT (32% reader 1, 37% reader 2) for the detection of extra enteric complications
Siddiki *et al*[68], 2009	United States	33 CD	Prospective blinded study, single centre, consecutive patients with suspected active small bowel CD April 2005-May 2008 Adult cohort (age range 20-63 yr)	MRE, CTE compared with ileocolonoscopy	No significant difference between sensitivity of MRE (90.5%) and CTE (95.2%) in detecting active small bowel CD In 8 cases (24%) MRE and CTE identified active small bowel inflammation not detected at ileocolonoscopy MRE significantly lower image quality score than CTE
Ippolito *et al*[69], 2009	Italy	29 CD	Prospective study, Single centre, symptomatic patients with proven CD and suspected relapse, recruited from outpatient clinic Adult and paediatric cohort (age range 14-70 yr) Mean age 43.8 yr	Contrast MRE and contrast multi-detector CTE	Complete agreement between MRE and CTE in classification of disease activity (k = 1) Good level of agreement between MRE and CTE for wall thickening and mucosal hyperenhancement (k = 1), comb (k = 0.9) and halo signs (k = 0.86) CTE superior to MRE in detecting fibrofatty proliferation (P = 0.045) MRE depicted higher number of fistulas than CTE but non-significant (P = 0.083)
Schreyer *et al*[70], 2010	Germany	53 CD	Retrospective study, Single centre, Patients with advanced CD and acute abdominal pain attending the emergency department Adult cohort	Conventional CT, MRE	No significant difference in image quality between CT and MRE No significant difference in diagnosis of small bowel inflammation between CT (69.4%) and MRE (71.4%) CT detection of lymph nodes significantly higher than MRE No significant difference in detection of fistulae (CT n = 25, MRE n = 27) or abscesses (CT n = 32, MRE n = 32)
Panés *et al*[41], 2011	Spain	N/A	Systematic review of 68 prospective studies, minimum 15 patients per study	US, CT, MRI for diagnosis of CD, assessment of disease extent and activity, detection of complications	Sensitivity for diagnosis of suspected CD and evaluation of disease activity: US 84%, MRI 93% Specificity for diagnosis of suspected CD and evaluation of disease activity: US 92%, MRI 90% CT similar accuracy to MRI for assessment of disease activity and extension. US accuracy lower for disease proximal to terminal ileum US, CT, MRI all high accuracy for detection of fistulas, abscesses, stenosis. US higher false positive for abscesses
Fiorino *et al*[43], 2011	Italy	44 CD	Prospective study, Single centre, consecutive patients with ileocolonic CD requiring endoscopic or radiological evaluation Enrolled 2006-2009 Adult cohort (> 18 yr) Mean age 44 yr	CTE and MRE to assess disease activity and complications in ileocolonic CD, using ileocolonoscopy as reference standard	MRE significantly superior to CTE in detecting internal strictures: sensitivity (92% *vs* 85%), accuracy (95% *vs* 91%), specificity (90% *vs* 51%) Overall no significant difference in sensitivity and specificity of MRE and CTE in localising CD, bowel wall thickening, bowel wall enhancement, enteroenteric fistulas, detection of abdominal nodes, perivisceral fat enhancement Per segment analysis, MRE significantly superior to CTE in detecting ileal wall enhancement, with higher sensitivity (93% *vs* 81%) and accuracy (88% *vs* 81%), but lower specificity (72% *vs* 81%). MRE significantly superior in localising rectal disease, with higher accuracy (93% *vs* 85%), specificity (100% *vs* 50,9%) but lower sensitivity (72% *vs* 81%)

Jensen *et al*[71], 2011	Denmark	50 CD	Prospective, multicentre study, patients with symptomatic pre-existing CD requiring small bowel imaging for treatment decisions	MRE and CTE compared with gold standard of ileoscopy or surgery	No significant difference between MRE and CTE for detection of small bowel CD MRE: sensitivity 74%, specificity 80% CTE: sensitivity 83%, specificity 70% No significant difference for detection of small bowel stenosis. MRE: sensitivity 55%, specificity 92%. CTE: sensitivity 70%, specificity 92%
Chatu *et al*[50], 2012	United Kingdom	143 CD	Retrospective study, single tertiary centre, all symptomatic patients with known or suspected CD who underwent SICUS retrospectively were reviewed June 2007-Dec 2010 Adult cohort Mean age 36 yr	SICUS compared with SBFT, CT, histological findings from ileocolonoscopy or surgery, and CRP, using final diagnosis as the reference standard	Sensitivity of SICUS in detecting active small bowel CD in known or suspected cases 93%, specificity 99%, positive predictive value 98%, negative predictive value 95% Agreement between SICUS with SBFT (k = 0.88), CT (k = 0.91), histological findings (k = 0.62), CRP (k = 0.07)
Pallotta *et al*[51], 2012	Italy	49 CD	Prospective study, consecutive patients, adult and paediatric CD who underwent resective bowel surgery Jan 2000-Oct 2010 Mean age 37.7 yr (Age range 12-78 yr)	Conventional transabdominal US and SICUS compared to intraoperative and histological findings to assess CD complications	SICUS ability to: Detect at least one stricture: Sensitivity 97.5%, specificity 100%, k = 0.93 Detect two or more strictures: Sensitivity 75%, specificity 100%, k = 0.78 Detect fistulas: Sensitivity 96%, specificity 90.5%, k = 0.88 Detect intra-abdominal abscesses: Sensitivity 100%, specificity 95%, k = 0.89
Qiu *et al*[44], 2014	China	290 CD	Systematic review with meta-analysis including six studies, all prospective with enrollment of consecutive CD patients	MRE and CTE in detecting active small bowel CD and complications	Pooled sensitivity MRE in detecting active small bowel CD: 87.9%, specificity 81.2% Pooled sensitivity CTE in detecting active small bowel CD 85.8%, specificity 83.6% No significant difference between MRE and CTE in detecting fistula, stenosis and abscesses.
Kumar *et al*[52], 2015	United Kingdom	67 CD	Retrospective study, Single tertiary centre. Adult cohort (age 18.8-68.9 yr) CD patients requiring resective bowel surgery within 6 mo of SICUS/ MRE investigation being performed June 2007-December 2012	SICUS and MRE compared to intraoperative findings	Sensitivity of SICUS and MRE in detecting: Strictures: 87.5%, 100% Fistulae: 87.7%, 66.7 Abscesses: 100%, 100% Bowel dilatation: 100%, 66.7% Bowel wall thickening: 94.7% and 81.8% Compared with surgery, high level of agreement of SICUS, MRE in: Localising strictures: k = 0.75, 0.88 Fistulae: k = 0.82, 0.79 Abscesses k = 0.87, 0.77 High level of agreement between SICUS and MRE in identifying stricturing disease (k = 0.84), number and location of strictures (k = 0.85), fistulae (k = 0.65), mucosal thickening (k = 0.61)
Aloi *et al*[53], 2015	Italy	25 CD	Single tertiary centre for paediatric IBD Paediatric cohort with known or suspected small bowel CD	MRE, SICUS, CE for diagnosis of small bowel CD	Jejunum: Specificity CE significantly lower (61%) than MRE. No significant difference in sensitivity: SICUS 92%, CE 92%, MRE (75%) Proximal and mid-ileum: Specificity CE significantly lower. No significant difference in sensitivity: MRE 100%, CE 100%, SICUS 80% Terminal ileum: Sensitivity of SICUS and MRE (94%, 94%) higher than CE (81%), CE more specific

CD: Crohn's disease; MRE: Magnetic resonance enterography; CT: Computed tomography; CTE: Computerised tomography enterography; SICUS: Small intestine contrast-enhanced ultrasonography; CE: Capsule endoscopy; BE: Barium enteroclysis; SBFT: Small bowel follow-through; US: Ultrasonography; MRI: Magnetic resonance imaging.

since produced more promising results. A German study of 23 patients with suspected IBD, comparing MRC using water enemas to colonoscopy findings, identified a sensitivity of 87% and specificity of 100% for detecting colonic inflammatory changes[47]. Recent studies have supported the reliability of diffusion weighted imaging MRC (DWI-MRC) for detecting colonic inflammation in UC, without the need for bowel preparation[48]. Advances in contrast media and DWI-MRI may increase the sensitivity and role of MRC in evaluating colonic inflammation in IBD, particularly in patients intolerant to colonoscopy. However, larger

scale comparative data is still required.

Trans-abdominal US and small intestine contrast-enhanced US

Trans-abdominal US has increasingly been favoured as a non-invasive imaging tool useful for the diagnosis of small bowel CD. It has advantages over SBFT in detecting extra-intestinal disease, is more cost effective and better tolerated than MRI, and avoids the radiation exposure of CT imaging. However, conventional trans-abdominal US is often limited by the presence of endoluminal gas and collapsed bowel walls, which may obscure pathology[49]. Administering oral contrast prior to performing US promotes bowel loop distension, improving bowel wall visualisation. As a consequence, small intestine contrast-enhanced US (SICUS) has emerged as a more accurate alternative to conventional US for the diagnosis and monitoring of small bowel CD[45,49].

A prospective Italian study by Parente *et al*[49] of 102 patients with CD, compared conventional US with SICUS for the diagnosis of CD and its intraluminal complications. Per-segment analysis revealed a superior diagnostic accuracy of SICUS in detecting small bowel CD. Indeed use of an oral anechoic contrast agent resulted in an increase in sensitivity from 91.4% to 96.1%[49]. SICUS was also more accurate than conventional US in detecting strictures and measuring the extent of small bowel involvement. Both conventional US and SICUS had a higher diagnostic accuracy than SBE in detecting fistulas, using intra-operative findings as the gold standard[49]. More recently, a United Kingdom-based study of 143 patients with suspected or known CD, found SICUS to have a similar diagnostic yield compared to SBFT and CT (k coefficient 0.88 and 0.91 respectively) for the detection of features of small bowel CD in routine clinical practice[50]. The sensitivity and specificity of SICUS for the detection of active small bowel CD was 93% and 99% respectively, with a positive predictive value of 98% and a negative predictive value of 95%. Furthermore, there was substantial agreement between SICUS and histology obtained at ileocolonoscopy or surgery (k = 0.62)[50].

SICUS may also have a role to play in the pre-operative assessment of CD. A prospective study by Pallotta *et al*[51] of 49 patients with CD, compared SICUS with intra-operative findings for the detection of small intestinal complications of CD. SICUS demonstrated high sensitivity and specificity for the detection of small bowel strictures (97.5% sensitivity, 100% specificity, k = 0.78), fistulas (96% sensitivity, 90.5% specificity, k = 0.88), and abscesses (100% sensitivity, 95% specificity, k = 0.89)[51]. Similarly, Kumar *et al*[52] compared SICUS and MRE in routine clinical practice with intra-operative findings in patients with CD requiring surgery. Correlating SICUS and MRE with surgery, there was a high level of agreement in localising strictures (k = 0.75, k = 0.88), fistulae (k = 0.82, 0.79) and abscesses (k = 0.87, 0.77)[52].

SICUS may be particularly well suited to investigating small bowel CD in children, where routine additional challenges include poorer tolerance to ileocolonoscopy (IC) requiring general anaesthetic, difficulty lying still for a time-consuming MRI, and increased sensitivity to ionising radiation. A recent prospective study by Aloi *et al*[53] compared MRE, SICUS and video capsule endoscopy in the evaluation of 25 children with suspected or known CD. Overall there was no significant difference among the three imaging modalities for the detection of active small bowel CD. Combining diagnostic imaging improved collective sensitivities, and combining SICUS with the serological marker C-reactive protein increased the specificity for the detection of CD from 89% to 100% in the jejunum, and from 79% to 100% in the distal ileum ($P < 0.05$)[53].

Preliminary data has suggested a role for power Doppler imaging in enhancing the diagnostic accuracy of conventional US and SICUS. Power Doppler US allows assessment of bowel wall vascularity, which has been shown to correlate well with disease activity in CD[54]. It can also aid in distinguishing between inflammatory and fibrotic stenosis[50].

Overall, SICUS has emerged as an accurate, well tolerated, radiation-free imaging tool for the assessment of small bowel CD. Limitations include inter-observer variability and difficulty interpreting and comparing images retrospectively given that it is a dynamic procedure. The diagnostic accuracy of SICUS is operator dependent and often thought to be dependent on experience. Although, in a 2005 Italian study SICUS performed by a inexperienced sonographer achieved superior diagnostic accuracy for assessing small bowel CD lesions compared to conventional trans-abdominal US performed by an experienced sonographer[55]. The results of a large multi-centre prospective study comparing MRE with US in CD patients are keenly awaited[56].

Contrast enhanced ultrasonography

Contrast enhanced ultrasonography (CEUS) is a new technique that involves the administration of an intravenous contrast agent, real-time, during ultrasonography. It allows more accurate evaluation of bowel wall vascularisation. Mural hyper-enhancement following contrast in CEUS has been shown to correlate well with bowel inflammation and allows grading of CD activity[57,58]. CEUS also has the potential additional benefit of better distinguishing between inflammatory and fibro-stenotic lesions, which can be difficult with conventional ultrasound[58]. CEUS does not require oral preparation, therefore it is well tolerated by patients and can be repeatedly performed to monitor disease activity. Limitations include the need for specific software, and increased procedure time.

Current studies suggest a role for CEUS in monitoring treatment response in CD, but further prospective studies are required to quantify how well CEUS correlates with endoscopic changes and SICUS[57].

CONCLUSION

Increased awareness of the cumulative exposure of IBD patients to diagnostic medical radiation is warranted, particularly given the potential for an increased risk of radiation-induced malignancy in patients exposed at a younger age. Creation of radiation diaries is a useful consideration to log total radiation exposures. MRI and SICUS are alternative, radiation-free imaging modalities, with proven diagnostic accuracy, and should be routinely considered for the diagnosis and evaluation of patients with small bowel CD wherever possible. More provision is needed for out-of-hours radiation-free imaging modalities to reduce the need for CT.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

Diet therapy for inflammatory bowel diseases: The established and the new

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Abstract

Although patients with inflammatory bowel diseases (IBD) have a strong interest in dietary modifications as part of their therapeutic management, dietary advice plays only a minor part in published guidelines. The scientific literature shows that dietary factors might influence the risk of developing IBD, that dysbiosis induced by nutrition contributes to the pathogenesis of IBD, and that diet may serve as a symptomatic treatment for irritable bowel syndrome-like symptoms in IBD. The role of nutrition in IBD is underscored by the effect of various dietary therapies. In paediatric patients with Crohn's disease (CD) enteral nutrition (EN) reaches remission rates similar to steroids. In adult patients, however, EN is inferior to corticosteroids. EN is not effective in ulcerative colitis (UC). Total parenteral nutrition in IBD is not superior to steroids or EN. The use of specific probiotics in patients with IBD can be recommended only in special clinical situations. There is no evidence for efficacy of probiotics in CD. By contrast, studies in UC have shown a beneficial effect in selected patients. For patients with pouchitis, antibiotic treatment followed by probiotics, like VSL#3 or Lactobacillus GG, is effective. When probiotics are used, the risk of bacterial translocation and subsequent bacteremia has to be considered. More understanding of the normal intestinal microflora, and better characterization of probiotic strains at the phenotypic and genomic levels is needed as well as clarification of the mechanisms of action in different clinical settings. A FODMAP reduced diet may improve symptoms in IBD.

Key words: Enteral nutrition; Parenteral nutrition; Probiotics; Fermentable oligo-, di-, and monosaccharides and polyols; Crohn's disease; Ulcerative colitis

Core tip: Over the last decades various dietary components like milk, fructose, salty foods and sweetened beverages have been implicated to play a role in the pathogenesis of inflammatory bowel disease (IBD), possibly by interacting with gut microbiota and the mucosal immune system. The role of nutrition in IBD is underscored by the effect of various dietary therapies. In paediatric patients with Crohn's disease enteral nutrition reaches remission rates similar to steroids. The use of specific probiotics in patients with IBD can be recommended only in special clinical situations. A FODMAP reduced diet may improve symptoms in IBD.

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INTRODUCTION

Approximately 2 million people worldwide suffer from inflammatory bowel disease (IBD), comprising Crohn's disease (CD), ulcerative colitis (UC) and pouchitis[1].

The exact pathomechanism of IBD is remains unexplained[1]. According to the literature, these diseases may result in part from the mucosal immune response to altered gastrointestinal microbiota in genetically susceptible individuals[1,2]. An abnormal mucosal barrier function in IBD may allow bacterial access to the lamina propria, triggering an inflammatory response[1], though additional environmental factors must be presumed to be involved in the aetiology of IBD[3].

IBD was first recognized as a major health issue in developed countries[4]. Many studies on children and adults in Western Europe and North America over the past 20 years have indicated that the incidence in IBD has increased to the extent that it is no longer a rare condition but affects up to 0.5% of the population[5-7] The incidence is also increasing in Japan or Eastern Europe, where it used to be uncommon[3,8]. Migrants from low prevalence countries take on the prevalence of their adopted high prevalence countries[9]. The incidence of CD appears to increase faster than UC[10]. This increased incidence is due in part to higher awareness and more reliable diagnosis of IBD, but the change to a Western lifestyle also suggests that environmental factors play a role in the development and progression of IBD[3,11].

Environmental factors that may be linked to the Western lifestyle have been implicated as predisposing to IBD[9]: these include smoking, appendectomy, better hygiene and use of oral contraceptives IBD[12,13]. One aspect of Western life style that has changed considerably in parallel with the emergence of IBD is diet[9,14,15]. The early increase in incidence up to the 1960s has been attributed to increased milk consumption[16] and more recently, other dietary components have been implicated. Over the last 20 years fructose intake has increased by more than 20% and intake of salty snacks, pizza, cereals and sweetened beverages has increased by nearly 50%[9]. As dietary antigens along with bacterial antigens are the most common types of luminal antigen, it is reasonable to suppose that dietary factors may play an important role in the pathogenesis of IBD[12], possibly by interacting with gut microbiota and the mucosal immune system.

The aim of therapeutic management is to achieve and maintain remission and prevent disease progression[1]. Pharmacological agents are the main pillar of therapy[17], but have side effects and some patients become refractory to them, necessitating surgery[1]. Nutrition plays a pivotal role in the clinical care of all patients with inflammatory bowel disease[11,18], and the efficacy of diet therapy in IBD was first investigated over 30 years ago[1,19]. In 1984, O'Moráin *et al*[20] reported that patients with acute Crohn's ileitis treatment with an elemental diet achieved remission rates comparable to those treated with corticosteroids, paving the way for an alternative to conventional therapy[18], though the mechanisms responsible are still not well understood[21]. Theories include improved nutritional status, reduced allergenicity of gut contents, avoidance of food additives as well as changes in the nature or quantity of gut bacteria[21]. Intestinal inflammation might be initiated by an aberrant response to the gastrointestinal microbiota, meaning that dietary substances and their metabolites might modify mucosal barrier function[1,22].

Nutrition in IBD as compared to controls

In recent decades a wide range of nutrients and their roles in the etiology of IBD have been investigated[11]. Persson *et al*[23] found an increased relative risk of IBD with consumption of fast food at least twice a week. Epidemiological studies suggest an increased risk of IBD associated with a high intake of the so-called "Western diet", which includes refined sugar and meat and animal fat, along with low fiber intake[14,21]. In contrast, a diet emphasizing vegetables, fruits, olive oil, fish, grains and nuts decreased the risk of pediatric IBD, especially CD[24].

As association of dietary factors and CD has been documented. These factors include the quantity and quality of fat intake, fast food ingestion, total protein and energy intake. Patients with CD consume more sugar or cereals than controls[9,25-28]. Geerling *et al*[29] found that CD patients consumed more carbohydrates than controls, but it was unclear whether this was

actually due to avoidance of other fat or protein sources. They also found that CD patients with a high Crohn's disease activity index (CDAI > 150) had a significantly higher carbohydrate intake than those in remission[29]. The literature is ambiguous on nutrients, as some studies did not confirm a significant association between high sugar intake and the incidence of CD[3,30].

A higher intake of linoleic acid, an n-6 polyunsaturated essential fatty acid found in red meat, various cooking oils and certain margarines, was associated with an increased risk of UC[4,31,32]. Linoleic acid is metabolized to arachidonic acid, which has pro-inflammatory properties and is increased in the mucosa of patients with UC[31].

Possible mechanisms for the association between IBD and nutrition

Possible mechanisms by which diet may cause IBD include a direct effect of dietary antigens, diet-induced alteration of gene expression, alteration of the composition of the enteric flora and an effect on gastrointestinal permeability or the immune system[11].

One hypothetical pathomechanism of CD is increased intestinal permeability, which might in turn increase exposure of the subepithelium to luminal pro-inflammatory molecules and microorganisms[9]. High luminal concentrations of short-chain fatty acids, due to bacterial metabolism of incompletely absorbed carbohydrates[33], can reversibly impair barrier function by inducing apoptosis of epithelial cells[9]. FODMAPs (fermentable oligo-di-monosaccharides and polyols) are a potential source of poorly absorbable carbohydrates[9], but a causal relationship between these foods and IBD remains unclear, as well as the potential confounding factor of socioeconomic status, which is related to food intake. In addition, the role of processing and cooking should be taken into account[34].

Kiss *et al*[35] have shown that some dietary components derived from vegetables interact with intestinal immune receptors influencing intestinal immunity. Vegetables of the Brassicaceae family (*e.g.*, broccoli, cabbage or Brussels sprouts) can activate the aryl hydrocarbon receptor (AhR), which is expressed ubiquitously in vertebrate cells[36]. AhR is highly expressed by intestinal intraepithelial lymphocytes and is involved in the defense against luminal attacks[37]. This receptor is down-regulated in the intestinal inflamed tissue of patients with IBD and activation of AhR can inhibit inflammation and colitis in the gut of mice[38]. The AhR system seems to be a link between external environmental stimuli and the immune system[39]. Dietary factors might interact with the AhR, which affects cytokine expression, synthesis of defensins, antimicrobial peptides and consequently also the microbial composition[36]. Activation of AhR by nutrients might have a beneficial effect in IBD. Clinical trials evaluating the stimulating effect of nutrition on the AhR are still missing.

ROLE OF GUT MICROBIOTA

The colonic microbiota are estimated to comprise 10^{14}[40,41]. Humans harbour 500 to 1000 different bacterial species in the gastrointestinal tract[42], with more than 90% representing *Firmicutes* and *Bacteroidetes*[42], with *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Spirochaetae* and *Verrucomicrobia* making up the rest[42]. At birth, the human gut is sterile and colonization by bacteria starts within the first hours of life[40]. The composition of the gut microbiome in infants varies greatly, depending on mode of delivery and infant feeding[43]. During childhood, a more stable, highly individual adult gut microbiome develops out of this diversity[40,44,45], with a symbiotic relationship forming between human host and his/her gut microbes[40]. This complex ecosystem protects against invasion by pathogens by successfully competing with them for nutrients and epithelial binding sites[46]. The human gut provides an ideal environment for the microbiota, which benefit the human host by fermenting indigestible food substances[40]. A balanced microbiota is important for maintaining the health of the host[40], but this balance may be upset by factors like host genetics, antibiotic treatment, intestinal inflammation or diet.

Along with food, microbiota provide the most common luminal antigens in the bowel, and these could influence intestinal inflammation[47]. Different dietary regimens might alter the composition of the gut microbiota and consequently affect the risk of IBD[34], but there are few data to evaluate this issue. In animal models, changes in fat and carbohydrate content in nutrition significantly affect gut microbiota[48]. When Turnbaugh *et al*[49] transfected germ-free C57BL/6J mice with human microbial communities and changed their diet from low-fat, high plant polysaccharide to high-fat, high-sugar "Western-style", their microbiota shifted within a single day. A study in interleukin-10 knockout mice has further shown that a mouse diet high in saturated fat increased the spontaneous rate of colitis as compared to a normal mouse diet[21]. A study in Japan noticed that people living in rural areas, eating typical Japanese food, have different faecal microflora than people living in urban areas, especially as to the number of Bifidobacteria[50]. These data suggest that some kinds of food might alter the intestinal microbiota population[21].

The role of the gut microbiota in inflammatory bowel disease has raised the interest in exchanging presumably abnormal microbiota in patients with IBD with fecal microbiota from healthy persons. However, the available evidence for this is still very weak. A recently published systematic literature review on the use of fecal microbiota transplantation (FMT) in IBD summarized 31 publications with 133 patients,

43% of whom had a Clostridium difficile infection. Improvement of symptoms was reported in 71% of patients with IBD. FMT may have the potential to be helpful in managing patients with IBD, but considerable further efforts are necessary to make this procedure a valid option for these subjects[51].

Microbiota, immune function and IBD

The human colonic microbiota plays a central role in inducing disorders of immune function and inflammation[52,53] and studies in recent decades have shown that bacteria are involved in the pathogenesis of IBD[53]. The association between *NOD 2/CARD15* gene polymorphisms and CD supports the link between IBD and gut microbiota. This gene has a role in bacterial peptidoglycan recognition and mice deficient in NOD2 show an increased susceptibility to bacterial infection through the luminal route[54-56]. Abnormalities of NOD2 in IBD patients might decrease tolerance to enteric bacteria with secondary inflammation of the gut[54]. In addition, animal models have shown that intestinal inflammation fails to develop when animals are kept in germ-free environment, supporting the role of the microbiota in the pathogenesis of IBD[57]. An imbalance in bacterial function so might play a role in the initiation of IBD[34]. Further, genetic studies have determined that many of the genetic risk alleles for IBD are involved in protecting the host from bacterial invasion of the gut[40]. These risk loci contain genes regulating the epithelial barrier or the innate immune response.

Alterations in the gut microbiome have been associated with IBD[40,58]. Ewaschuk *et al*[59] found that *Bacteroides* spp., *Enterococcis faecalis*, *Enterobacter cloacae*, intestinal *Heliobacter* spp., *Fusobacterium* spp., adherent/invasive *Escherichia coli* strains, *Eubacterium* and *Peptostreptococcus* spp. seem to be harmful intestinal microbes. In contrast, *Lactobacillus* spp., *Bifidobacterium* spp., *Streptococcus salivarius*, *Saccharomyces boulardii* (*S. boulardii*), *Clostridium butyricum*, *Ruminococci* and *Escherichia coli* (*E. coli*) Nissle 1917 seem to be beneficial[59]. Most individuals with IBD, especially CD, are characterized by dysbiosis, in which one or a few potentially harmful microorganisms are dominant[53,60]. In patients with IBD, decreased *Firmicutes*, increased *Proteobacteria* as well as changes in *Bacteroides* compared to healthy controls have been detected[34,61-65]. It has been demonstrated that recurrence of inflammation after ileal resection depends on the exposure of the neoterminal ileum to faecal contents[66,67]. However it is not known which component of faeces triggers this inflammation[40].

NUTRITION AS A THERAPEUTIC OPTION

Although the development of highly active drugs like anti-TNF alpha antibodies has changed the short-term prognosis of severe IBD, there is still a need for low-risk alternative approaches or adjuvant therapies[68]. A number of trials have investigated the efficacy and mechanisms of action of diet in IBD. Historically, nutrition has been seen as an adjunctive therapeutic option and not as a source of therapeutic strategies on its own right[68]. Because of the delivery of high loads of specific nutritional compounds that can affect different targets, nutrition is now not only used as a supportive measure but has also been suggested as primary treatment[18] to induce or maintain remission.

Many studies have shown that clinical remission and mucosal healing in IBD can be achieved with different nutritional regimes. Studies evaluating the effect of enteral nutrition (EN), total parenteral nutrition (TPN), probiotics or FODMAP reduced diet in IBD have been published, but due to the heterogeneity of study protocols, there are as yet no recommendations for routine clinical practice[1]. This review examines concepts of diet therapy in IBD concerning EN, TPN, probiotics and FODMAPs.

Enteral nutrition

Enteral nutrition in CD: The literature includes numerous studies analysing the effect of EN on IBD. EN can be given via the nasogastric tube or by mouth. Generally it is offered in two forms: first, as an elemental diet, containing nutrients in simple forms such as amino acids, mono- or oligosaccharides and medium-chain triglycerides that require little or no digestion prior to absorption[69]; second, as polymeric diets, containing whole proteins and carbohydrates as hydrolysates of starch, which are mostly more palatable[69].

In 2006, the European Society for Parenteral and Enteral Nutrition published guidelines on the role of EN in IBD[70]. In general, EN is indicated in undernourished patients with CD or UC to satisfy nutritional needs[4]. Additionally, in children with active CD, EN is the first line therapy to achieve remission. In adults, however, EN might be a therapeutic option only if treatment with corticosteroids is not tolerated[4]. According to the guidelines of the European Crohn' s and Colitis Organisation ECCO, EN may be an option together with other medications to maintain remission in selected patients[71].

There are meta-analyses and a Cochrane review of randomized controlled trials investigating the effect of EN in CD[72-75]. These studies have mostly shown that EN as sole nutrition can induce clinical remission and mucosal healing in CD[21]. Many trials have evaluated the effect of EN in paediatric CD with the aim of avoiding corticosteroids with their risk of growth suppression[69].

Induction of remission: Paediatric studies give strong support to the role of EN, showing efficacy similar to corticosteroids and better improvements in

growth and mucosal healing[76-81]. In a meta-analysis of paediatric trials comparing the outcome of EN *vs* corticosteroids, there was no significant difference between the two groups[82]. Remission rates were higher with total EN than with partial EN (10/24 *vs* 4/26, RR = 2.7, 95%CI: 1-7.4)[4,82]. Day *et al*[78] evaluated the effect of EN in 27 children with active CD who received polymeric feed for 6-8 wk and no other medication. At the end of the study 80% of the newly diagnosed patients and 58% of the known CD patients were in remission[78].

In most paediatric IBD centres EN is given exclusively for 6 to 8 wk, mostly (90%) as polymeric formulas[21,83]. The mode of reintroduction of food after exclusive EN varies widely, but usually involves an initial low-fibre diet (26%) or gradual re-introduction of normal food, as the formula volume is decreased (56%)[21] .

In adult patients, a Cochrane meta-analysis of six RCTs including 192 adult patients treated with EN and 160 with corticosteroids indicated that EN is less effective than corticosteroids in inducing remission of active CD[75], but this conclusion was based on an intention-to-treat analysis, and patients who successfully completed EN achieved remission rates comparable to those receiving corticosteroids[4,18]. Compared to placebo treated patients with response rates of 20%-30%, patients with EN reached remission rates up to 60%[11,75,84,85]. Lochs *et al*[86] compared the effect of EN as sole therapy to a treatment with steroids and sulfasalazine in patients with active CD (CDAI > 200). After 6 wk, 53% in the EN group and 79% in the drug-treatment group achieved remission (CDAI decreased by 40% or at least 100 points). In this study EN was less effective than a combination of steroids and sulfasalazine for active CD.

Maintenance of remission: EN might be used not only to achieve but also to maintain remission[87,88]. Several studies in adults and children have assessed the efficacy of EN in maintaining medically or surgically induced remission[89-92]. These studies have shown significantly lower recurrence rates in patients treated with EN than in those on a normal diet[4]. Mucosal cytokines like interleukin-1 beta, interleukin-6 and tumour necrosis factor alpha were significantly lower in patients receiving EN than in those on a normal diet[93], suggesting that EN might alleviate mucosal inflammation and so promote remission[4]. Takagi *et al*[91] randomized 51 CD patients who had recently achieved remission, 26 to receive half their calorie intake as EN and 25 to have a free diet. Over a mean follow-up of 11.6 mo, the relapse rate was 34.6% in the "half enteral" group and 64% in the free diet group (relapse was defined as either a CDAI score of more than 200, or the need for therapy to re-induce remission). This study concluded that EN given as 50% of calories seems to be effective in maintaining remission[21]. Hanai *et al*[94] compared the effect of 6-mercaptopurine (6-MP), an elemental diet and no therapy in patients on CD maintenance. After 24 mo, the clinical remission rates were 60, 47 and 27% for 6-MP, elemental diet and the control group, respectively. The remission rates in the 6-MP and elemental diet groups were significantly higher than in the control group. There was no significant difference between the 6-MP and the elemental diet group[94].

In another prospective study, EN significantly reduced clinical as well as endoscopic recurrence within 1 year in CD patients as compared to patients without any nutritional therapy[93]. This study included 40 adult patients with CD who achieved clinical remission (CDAI < 150). Of them, 20 received continuous elemental diet infusion during the night and a low-fat diet during the day, while the other 20 received neither nutritional therapy nor food restriction[93]. Twenty-five percent in the EN group and 65% in the non-EN group had a clinical relapse during the 1-year observation period, with clinical relapse defined as CDAI > 150.

Yamamoto *et al*[88] evaluated the effect of enteral nutrition on maintenance of clinical remission in patients with CD receiving infliximab (IFX) maintenance therapy. This study showed that concomitant enteral nutrition during IFX maintenance therapy does not significantly increase the maintenance rate of clinical remission in CD patients[88].

Mucosal healing: EN may have a positive effect on mucosal healing[76]. Yamamoto *et al*[95] have shown reduced mucosal cytokine production in adults with CD treated with elemental diet. Besides, some paediatric studies have shown that EN is more effective than corticosteroids in inducing mucosal healing[96,97]. Since along with symptomatic improvement, the main target in the treatment of CD is mucosal healing, this advantage of EN over corticosteroids might be considered in therapeutic decision-making[4,98].

Comparison of different EN formulas: Several studies have compared the effect of different types of enteral formulas in the management of CD: elemental, semi-elemental or polymeric diet[4]. A Cochrane meta-analysis of 10 trials did not show any difference between elemental diet and non-elemental diet[75]. There is no systematic review that shows that one type of enteral formula is better than others[4,18]. This might imply that the therapeutic effect of EN is independent of the type of nitrogen source[18]. There was also no difference in efficacy when EN was compared with low fat or high fat diet, and when foods with high-omega-3 *vs* high-omega-6 fatty acid content were compared[21,75,99]. Whole protein (polymeric) food works comparably well to amino-acid-based food and is generally less hyperosmolar[21]. In summary, the quantity or type of fat and protein does not affect the therapeutic potential of enteral nutrition[75,100-103]. There is no association between the efficacy of EN and locations of CD[21], but the validity of a meta-analysis

covering different feeds would be uncertain because of their variety composition[21].

Potential mechanisms: The mechanism by which EN improves CD is unclear. Hypotheses include altered or reduced gut microbiota, avoidance of long-chain fat, which impairs macrophage function, and avoidance of other harmful components of normal food, like emulsifiers or nano-particles as additives[21]. Malnutrition, which is often a problem in IBD patients, can affect immune function and wound healing. When correction of nutritional status improves wound healing, reduced gut permeability could enhance mucosal healing as well[69].

Gut permeability is thought to play an important role in CD. Abnormalities in tight junctions between enterocytes increase luminal antigen uptake, which might contribute to inflammatory activity[57]. Therapy with an elemental diet was shown to reduce intestinal permeability[104], and the literature also suggests that there is a change in the faecal microbiome following exclusive EN[105,106].

A study with paediatric CD patients looked at the impact of exclusive EN on gut microbiota, which showed reduced diversity and an increase in *Protobacteria*[21,105,107]. Leach *et al*[105] compared the bacteria in the stool in patients with CD under exclusive enteral nutrition to a group of healthy controls under a regular diet. At the start of the study, the diversity of bacteria in the two groups was similar but after 8 wk, the patients treated with exclusive EN had significantly less bacterial diversity than the control group.

Long-term perspective of enteral nutrition: Compared to corticosteroids, EN has no long-term adverse effects[18], though there are many other factors that can potentially influence its efficacy[4]. The palatability of feeds and the length of time without solid food, as well as the social inconvenience, contribute to high dropout rates and reduced patient compliance[108]. Additionally, the high cost of enteral formulae has to be considered. A major problem of EN as primary therapy for CD is the high relapse rate, approximately 50% within 6 mo, when patients return to normal diet[21,103]. There is evidence to advocate an exclusion and re-introduction diet following successful induction of remission with EN, rather than an immediate return to a normal diet[11,18]. At 2 years, the remission rate was 59%[18,109] when patients were weaned off EN for 2-4 wk, and, before normal diet was re-introduced, given a limited range of foods generally tolerated by CD patients. After achieving clinical remission, patients with good compliance might receive a half elemental diet with approximately half of their calories derived from an elemental diet[18]. A return to normal feeding nonetheless often leads to relapse[110] and a lower efficacy of dietary therapy in distal disease (colonic/perianal) has been described[111].

Enteral nutrition in UC

There is no evidence that EN is an effective therapy for active UC[21]. One prospective randomized trial compared the effect of TPN and total EN as an adjunct therapy in patients with acute UC on intensive steroid therapy[112]. Remission rates as well as need for colectomy were similar in these two groups.

TOTAL PARENTERAL NUTRITION

Since dietary antigens may be important stimulants for the mucosal immune system, bowel rest with total parental nutrition (TPN) has been considered as a therapeutic option in IBD. The aim of TPN as primary therapy for IBD is to achieve bowel rest, to correct nutritional deficits[11,18,113] and to remove antigenic mucosal stimuli[110]. Various studies have analysed effect of TPN and in the 1980´s especially, TPN was used to treat patients with moderate to severe CD[40].

Müller *et al*[114] prospectively evaluated the effect of TPN in 30 patients with CD, whereby 83% achieved remission, but relapse was common. Surgery could be avoided in 25 of 30 complicated CD patients on 3 wk of inpatient TPN followed by 9 more weeks at home[114]. Greenberg *et al*[115] compared the effect of TPN, partial parenteral nutrition (PPN) with supplementary nutrition with a defined formula *via* NG tube, or PPN with supplementary normal diet. There were no significant differences in the remission rates of 71% in the TPN group, 58% in the PPN with defined formula diet group and 60% in the group with PPN and normal diet[115]. Ostro *et al*[116] evaluated the effect of TPN in 100 patients with CD refractory to conventional medical management. In their study, 90 patients received complete nutrient replacement and 10 received protein-sparing therapy; Seventy-seven patients achieved clinical remission. The remission rate was not influenced by the location of the intestinal involvement. Additionally, TPN was shown to play a role in the healing of post-operative enterocutaneous fistulas arising from surgical anastomosis or complicated fistulas in CD[18,113,117]. When TPN and EN are compared, TPN is associated with higher costs and significant risks including line sepsis and should be restricted to patients who cannot take adequate nutrition enterally[21].

TPN had no effect on severe acute UC in two short-term studies of patients with severe disease[118,119].

PROBIOTICS

There is growing evidence for an association between IBD and an alteration in the gut microbiota but due to the complexity of the gut microbiota, research on this is still in its early stages. Studies have shown a disbalance in the gut between protective *vs* harmful intestinal bacteria[120] with, *e.g.*, an increase in mucosa-associated Escherichia coli and a reduction

in bifidobacterium and lactobacillus species[21,120]. Strategies modulating this dysbiosis might be a therapeutic option in IBD[33]. Antibacterial treatment has been used, but with limited effect[120]. Probiotics may improve intestinal microbial balance, enhancing gut barrier function and improving local immune response[59]. Probiotics are live microorganisms, which when administered in adequate amounts, confer a health benefit on the host[121]. Their effects are strain specific, so that comparisons and meta-analyses of studies using different probiotics are problematic.

Bacteria associated with probiotic activity like lactobacilli or bifidobacteria have been used as well as non-bacterial organisms such as *S. boulardii*[122], but it is a challenge to manipulate the highly individual gut microbiota. Potential mechanisms of probiotics are competitive interactions with the gut microbiota, production of antimicrobial metabolites, and interaction with the epithelium or immune modulation[122,123]. Cells involved in both the innate and adaptive immune responses, like B cells, T cells and dendritic cells as well as macrophages, might be affected[120,124,125]. Probiotic bacteria are able to antagonize pathogenic bacteria by reducing luminal pH[126,127] and inhibiting bacterial adherence and translocation[123]; they can also produce antibacterial substances and defensins[120,128]. For example, invasion of an epithelial cell line by invasive *E. coli* isolated from patients with CD was prevented by pre- or co-incubation with *E. coli* Nissle 1917[129]. Pre-treatment of IL-10 deficient mice with *Lactobacillus reuteri* and *L. casei* can reduce Heliobacter hepaticus-induced colitis[130]. A decrease in mucosal secretion of inflammatory cytokines was shown to be induced by *E. coli* (Nissle 1917) in models of experimental colitis[131]. Probiotics also influence cell-cell interactions and stability through modulation of intestinal barrier function[120]. Alterations in mucus, chloride secretion or changes in tight junction protein expression by epithelial cells might be mechanisms for improved gut mucosal barrier function[120,132].

There are no human data showing any effect of probiotics on dysplasia or colon cancer; however, in animal studies probiotics also seem to reduce the progression from inflammation to dysplasia and finally to colon cancer[130]. Oral administration of Lactobacillus salivarius UCC118 was shown to reduce the incidence of colon cancer as well as the severity of mucosal inflammation in IL-$10^{-/-}$ mice *vs* placebo[130]. Oral administration may not be required for certain probiotic effects: Il-$10^{-/-}$ mice had fewer proinflammatory cytokines after subcutaneous injection of *L. salivarius* UCC18[133].

Consequently, probiotics might improve IBD by regulating the inflammatory response or modulating gut microbiota composition. Many studies have tried to determine the effect of various probiotics in IBD and there will surely be more to come.

Probiotics in CD

There is no strong scientific evidence to justify the use of any of the probiotic strains that have been tested in the past in the management of CD[134,135]. The ECCO guidelines do not recommend probiotics to maintain remission in adult or paediatric CD patients[71]. The use of probiotics in patients with CD has produced ambiguous results and the available trials are small, with very few double blind, randomized, controlled trials[54].

The only positive study, by Guslandi *et al*[136], found that the yeast *S. boulardii* had an effect in CD. Thirty-two patients with CD in remission were treated with either mesalazin 3 g per day alone or mesalazin 2 g per day with *S. boulardii*. In the group treated with S. boulardii, significantly fewer patients relapsed than in the mesalazin only group (6.25% *vs* 37.5%, $P < 0.05$).

Fujimori *et al*[137] treated 10 patients with active CD unresponsive to 5-ASA or steroids with a therapy consisting of Bifidobacterium and Lactobacillus and the prebiotic psyllium for longer than 12 mo. Six patients achieved remission, 1 showed partial remission and there were 3 nonresponders. There were no significant differences when C-reactive protein and erythrocyte sedimentation values were compared before and after therapy. Malchow *et al*[129] treated 28 patients with active CD with prednisone and either E. coli Nissle or placebo. *E. coli* Nissle 1917 is a non-pathogenic *E. coli* that colonizes the intestine and inhibits the growth of enteropathogenic and other enteric bacteria[54]. This probiotic bacterium develops antagonistic activity against enterobacteria such as *Salmonella enteritidis*, *Shigella dysenteriae*, *Yersinia enterocolitica* and *Vibrio cholera*[138,139]. In this study, *E. Coli* Nissle was given in an increasing dosage over 24 d to a final dose of 5×10^{10} bacteria per day for one year. There was no statistically significant difference between the two groups in the time needed to induce remission[129]. In a placebo-controlled randomized study in children with CD treated with *Lactobacillus GG vs* placebo, there was also no significant difference in remission maintenance[140]. Seventy-five children were randomized to receive either *Lactobacillus GG* or placebo for 2 years, and the study concluded that *Lactobacillus GG* does not prolong time to relapse in children with CD when given as an adjunct to standard therapy. Schulze *et al*[138] determined the effect of *Lactobacillus GG vs* placebo in a controlled trial including 11 patients with mild to moderate CD. These patients received antibiotics (ciprofloxacin, metronidazole) for 2 wk, a tapering steroid regime over 12 wk and after 1 wk of antibiotic therapy they were randomized to receive either *Lactobacillus GG* or placebo for 6 mo[141]. The two groups did not differ as to relapse during the study period and mean time to relapse.

Recurrence of CD after surgical resection: For 1 year, Prantera *et al*[142] treated 45 patients who had undergone a curative ileocecal resection with either *Lactobacillus GG* or placebo but saw no benefit from the probiotic therapy. Marteau *et al*[143] studied the effect of *Lactobacillus johnsonii* (*L. Johnsonii*) in 98 patients with CD who had undergone a resection of less than 1 m of small bowel within 21 d prior to study enrolment. Patients were randomized to receive either *L. Johnsonii* or placebo for 6 mo, with no difference between the two groups. Van Gossum *et al*[144] also studied the effect of *L. Johnsonii vs* placebo in a 3-mo trial and again with the same result of no difference between the two groups.

Probiotics in UC

There are data that suggest that certain strains of probiotics are effective in the management of UC[145,146]. Tursi *et al*[147] studied the effect of the probiotic mixture VSL#3™. This contains 450 billion colony forming units of 8 lactic acid bacteria (*B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. casei*, *L. delbrueckii*, *L. plantarum* and *Streptococcus salivarius subsp. thermophilus*). Seventy-one patients were treated with this probiotic for 8 wk and compared to an untreated control group of 73 patients[147]. VSL#3 ™ was significantly superior to placebo in reducing the activity of mild-to-moderate UC. Other factors like the reduction in rectal bleeding as well as the reintroduction of remission in relapsing UC patients after 8 wk of treatment did not reach statistical significance[147]. Bibiloni *et al*[148] treated 34 patients with active UC unresponsive to conventional therapy with VSL#3™ daily for 6 wk and achieved a response rate up to 77%.

Twenty-four patients with mild to moderate UC flares were treated with *S. boulardii* for 4 wk and showed a response rate of 68%[149]. Zocco *et al*[150] evaluated the effect of *Lactobacillus GG* in a randomized study of 187 patients with quiescent colitis. For 12 mo they received either *Lactobacillus GG* alone, *Lactobacillus GG* and mesalazine 2400 mg/d or mesalazine 2400 mg/d alone. They concluded that Lactobacillus GG has efficacy similar to mesalazine in maintaining remission in UC. After 12 mo of treatment, remission was maintained of 85% in the Lactobacillus GG group, 80% in the mesalazine group and 84% in the combined treatment group.

Ishikawa *et al*[151] investigated the effect of daily Bifidobacteria supplemented fermented milk, (containing *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Lactobacillus acidophilus*), *vs* standard therapy with 5-ASA and steroids in 20 patients for 12 mo. The probiotic group had significantly fewer disease exacerbations than did the placebo treated control group.

Kruis *et al*[152] evaluated the effect of *E. coli* Nissle 1917 *vs* mesalazine 1500 mg per day in a 12-wk randomized trial with 103 patients with UC in remission. Relapse rates were similar in both groups, supporting the use of E. coli Nissle 1917 as a non-toxic treatment for ulcerative colitis. This result was confirmed by a larger study of the same group[139]. Rembacken *et al*[153] evaluated the effect of *E. coli* Nissle in maintaining remission in 83 patients with active UC at the start of the trial. At the beginning of the study patients received standard therapy to induce remission and were then randomized to either *E. coli* Nissle 1917 or mesalazine. There was no difference concerning relapse rates between the two groups.

Unlike other probiotics, the ECCO guidelines recommend *E. coli* Nissle as an effective alternative to 5-ASA for maintaining remission in UC[154].

Pouchitis

The most convincing data so far on the effect of probiotics in IBD have been found in the treatment of pouchitis[131]. The ECCO guidelines recommend probiotics as a therapeutic option for maintaining antibiotic-induced remission in recurrent pouchitis in pediatric UC[155]. Several probiotic strains are beneficial in preventing and treating pouchitis after surgery for UC[59].

Pouchitis is an idiopathic inflammatory disease of the ileal pouch that occurs in 15%-53% of UC patients following total abdominal colectomy with ileal pouch-anal anastomosis[54,156]. As faecal stasis with immunologic reactivity seems to be important in the pathogenesis of pouchitis, several studies evaluated the effect of probiotics in this disease. In a study with 40 patients with chronic relapsing pouchitis, Gionchetti *et al*[157] compared the effect of VSL#3 and placebo for 9 mo following one month's antibiotic therapy with rifaximin and ciprofloxacin. Eighty-five percent in the probiotic group stayed in remission *vs* 0% in the placebo group, as was confirmed by an international multicentre study[158]. In a different group of 31 patients with antibiotic dependent pouchitis, Shen *et al*[159] studied the effect of VSL#3. After 8 mo, 25 patients (71%) stopped therapy because of recurrence of symptoms or side effects.

Gionchetti *et al*[160] evaluated the effect of prophylactic probiotic therapy to prevent pouchitis. When 40 patients were randomized within a week after surgery to receive either VSL#3 or placebo for 12 mo[160], 10% in the probiotic group developed acute pouchitis compared to 40% in the placebo group. The VSL#3 treated group also showed a significantly lower stool frequency than the placebo group.

Similar results were shown in a study by Gosselink *et al*[161] with *Lactobacillus GG* in which 39 patients treated with Lactobacillus GG were compared to a placebo group for 3 years. In the probiotic therapy group significantly fewer patients developed pouchitis within those 3 years than in placebo group; however there is no current evidence that early intake of

prebiotics or probiotics can protect against the development of pouchitis, or even of IBD. Kuisma *et al*[162] evaluated the effect of Lactobacillus GG *vs* placebo in 20 patients in the treatment of active pouchitis for 3 mo and found no improvement in the probiotic group as compared to the control group.

Safety aspects

Although probiotics are considered safe, there have been case reports of bacteraemia and endocarditis associated with probiotic therapy[163]. In addition, the antibiotic resistance transferred from the probiotic bacteria to other bacteria in the gastrointestinal tract might also be of clinical relevance, and the risk of translocation of probiotics across the inflamed colonic mucosa in severely ill IBD patients has to be considered[54]. As mentioned above, the manipulation of the gut microbiota presents a challenge and the probiotic strain, dose, and frequency as well as the duration of the probiotic therapy have to be defined[120], taking into account that the highly individual variety of bacteria in the gut might cause bacteria-host interactions[120]. Further studies have evaluated the effect of nonviable bacteria. Since some of the beneficial effects of probiotics might be mediated by their DNA, live bacteria might not be needed at all[131].

FODMAP

Along with increased fast food and total protein and energy intake fructose consumption has also increased dramatically. Since the incidence of IBD has also increased in recent decades, an association between ingestion of incompletely absorbed fermentable carbohydrates (FODMAPs) and IBD has been postulated[9]. In the 1980s and 1990s, evidence was building for the role of poorly absorbed short-chain carbohydrates in the induction of gastrointestinal symptoms. These incompletely absorbed carbohydrates and polyols are summarized in the acronym FODMAPs. They include fructo-oligosaccharides (found in wheat, onions, legumes), lactose (found in milk and milk products), fructose (found in apples and many other fruits and vegetables, or honey), galactans (found in legumes) and sorbitol (found in stone fruits, artificial sweetener)[164]. Ingested FODMAPs are poorly absorbed in the small intestine. Due to their molecular size and osmotic effect, they draw fluid into the small and large bowel lumina[33,165]. FODMAPs can also induce gastrointestinal symptoms when they are fermented by intestinal bacteria and produce large amounts of gas[33,164,166].

Several trials have demonstrated the effect of a FODMAP-reduced diet in the treatment of irritable bowel syndrome (IBS). This diet restricts FODMAPs, as in wheat, onions, beans, many fruits and vegetables, and sorbitol and other sweeteners[21,167]. Dietary studies have demonstrated that reduction of FODMAPs reduces symptoms in up to 50% of patients[164]. In one case, the effect of a FODMAP reduced diet was determined in a placebo-controlled, cross-over rechallenge in patients with IBS[168]. Patients had fewer symptoms on the FODMAP reduced diet and symptoms recurred in 70%-80% of them when FODMAPs were reintroduced.

As 57% of patients with CD and 33% of patients with UC experience IBS-like symptoms[169,170], a FODMAP reduced diet might also be a therapeutic option in IBD[9,164]. In addition to relief from IBS-like symptoms in IBD, there are three observations that support the hypothesis that FODMAPs may also be involved in the pathogenesis of IBD[9]. First, the intake of FODMAPs in general and fructose specifically have increased in Western societies in recent decades. Second, there is an association between increased intake of sugars and the development of CD[9]. Third, excessive intake of FODMAPs creates conditions in the bowel like increased intestinal permeability that may predispose to CD[9]. To follow this up, studies have investigated the effect of a FODMAP reduced diet in IBD[164]. Barrett *et al*[165] treated 12 patients with ileostomy with either a low or high FODMAP diet for 4 d. Due to the osmotic effect[33] of FODMAPs, there was a 20% increase in ileal effluent on the high FODMAP diet as compared to the low FODMAP diet. It has been suggested that an increased inflow of FODMAPs into the distal small intestinal and proximal colonic lumen might underlie susceptibility to Crohn's disease. The passage of FODMAPs and their subsequent rapid fermentation might lead to expansion of bacterial populations with a secondary increase in intestinal permeability. Croagh *et al*[171] retrospectively determined the effect of a FODMAP reduced diet in patients with IBD. Seventy percent of patients who received instruction on a FODMAP reduced diet remained adherent to diet after 3 mo and reported a significant improvement of symptoms like pain, bloating, and diarrhea[171].

The role of bacteria in mediating the effects of FODMAP on intestinal inflammation was investigated by Zhou *et al*[172] in mice treated with low or high FODMAP diet or regular chow for 2 wk. Mice receiving the high FODMAP diet developed dysbiosis in the gut as well as mucosal inflammation and impaired intestinal permeability[172]. Pedersen *et al*[173] investigated the effect of a FODMAP-reduced diet in 89 patients with IBD (CD: 28 and UC: 61) suffering from IBS symptoms. They found a significant reduction in IBS-like symptoms in patients with IBD and a further reduction in disease activity for UC. In this study 70% of IBD patients with IBS symptoms were dysbiotic at the start of the diet and 50% after 6 wk on the diet. There were no significant changes in the microbiota in these patients after the 6 wk of reduced FODMAP diet[174].

It must, however, be borne in mind that most of the studies on the effect of a low FODMAP diet in IBD

Table 1 Summary of effects of nutritional interventions in inflammatory bowel disease

	Crohn's disease			Ulcerative colitis			
	Induce remission	Maintain remission	Postop.	Induce remission	Maintain remission	Postop.	Pouchitis
Enteral nutrition	Children: ++ Adults: +	++	+	No effect	No effect	No effect	Not tested
Total parenteral nutrition	+	No effect	Not tested	No effect	No effect	No effect	Not tested
Probiotics	No effect	No effect	No effect	+	+	Not tested	++
Low-FODMAP- diet	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested

are limited due to their retrospective and uncontrolled nature and lack of objective data on inflammatory changes associated with dietary intervention[14]. The FODMAP diet also greatly restricts consumption of many fruits and vegetables[175] and recurrence of symptoms after reintroduction of FODMAPs has to be expected.

CONCLUSION

Pharmacological therapy remains the mainstay of treatment of IBD. However, patients with IBD have a strong interest in dietary modifications as part of their therapeutic management. Unfortunately, dietary advice plays only a minor part in published guidelines for the management in IBD. The scientific literature shows that dietary factors might influence the risk of developing not only IBD but also intestinal mucosal inflammation. In addition, diet may serve as a symptomatic treatment for IBS-like symptoms in IBD. A "Westernized diet" rich in animal fat and protein and low in fiber may increase the risk of IBD. Dysbiosis induced by nutrition contributes to the pathogenesis of IBD.

Table 1 shows a summary of the effects of nutritional interventions in IBD. Due to the lack of large prospective controlled studies, no strong recommendations can be made at this point. The different protocols of many studies evaluating the effect of nutritional interventions in IBD, like EN, TPN, probiotics or a FODMAP reduced diet, are not comparable. Still, the role of nutrition in IBD is underscored by the effect of various dietary therapies, especially in paediatrics. In paediatric patients with CD, EN is an accepted therapeutic option. In this group EN has been shown to reach remission rates similar to steroids. Although some studies have shown similar benefits in adult patients with CD treated with EN compared to corticosteroids, a number of meta-analysis have shown that in adult patients, EN is inferior to corticosteroids. The protein or fat content of enteral formulae does not seem to affect clinical outcome. EN is not a successful therapeutic option in UC.

The efficacy of TPN in IBD is not greater than that achieved in other trials with steroids or EN.

The use of specific probiotics in patients with IBD can be recommended only in special clinical situations. There is no sound evidence to justify the use of probiotics in the management of CD. By contrast, studies on UC have shown a beneficial effect in selected patients. For patients with pouchitis, antibiotic treatment followed by probiotics, like VSL#3 or Lactobacillus GG, is effective in maintaining remission, though when probiotics are used, the risk of bacterial translocation and subsequent bacteremia has to be considered. Fulfillment of the therapeutic potential of probiotics requires more understanding of the normal intestinal microflora, and better characterization of probiotic strains at the phenotypic and genomic levels is needed as well as clarification of the mechanisms of action in different clinical settings. A FODMAP reduced diet may improve symptoms in IBD. More randomized controlled trails are necessary to evaluate the efficacy of these diet forms in IBD.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

Role of regulatory T cell in the pathogenesis of inflammatory bowel disease

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Abstract

Regulatory T (T_{reg}) cells play key roles in various immune responses. For example, T_{reg} cells contribute to the complex pathogenesis of inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis during onset or development of that disease. Many animal models of IBD have been used to investigate factors such as pathogenic cytokines, pathogenic bacteria, and T-cell functions, including those of T_{reg} cells. In addition, analyses of patients with IBD facilitate our understanding of the precise mechanism of IBD. This review article focuses on the role of T_{reg} cells and outlines the pathogenesis and therapeutic strategies of IBD based on previous reports.

Key words: Inflammatory bowel disease; Regulatory T cell; Animal model; Therapy

Core tip: We review the types and functions of regulatory $CD4^+$ T cells (T_{reg} cells) and describe their roles in the pathologies of the inflammatory bowel diseases, *i.e.*, Crohn's disease and ulcerative colitis. We have paid particular attention to the use of animal models and human studies to elucidate the mechanisms by which T_{reg} cells influence these diseases and have provided an overview of the potential uses of these cells in therapeutic strategies.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a term that encompasses two major forms of chronic inflammatory intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC)[1-3]. Both of these chronic inflammatory diseases feature a typical time of onset during young adulthood and a lifelong course characterized by periods of remission and relapse[4,5]. The pathogenesis of IBD is well known to be complex because of various immunological, environmental, host genetic, and bacterial factors, and the complicated regulatory mechanisms associated with mucosal immunity can also influence the onset or development of IBD[6-8]. Many reports of various animal models of IBD have revealed cellular or molecular mechanisms of IBD and the recent use of anticytokine agents, such as antitumor necrosis factor-α (TNF-α), interleukin (IL)-6R, or IL-13 antibodies, has achieved progress in healing mucosal damage associated with IBD[9,10].

Regulatory T (T_{reg}) cells are known to plays a key role in the pathogenesis of IBD, as well as in other autoimmune disorders or allergies[11-14]. Considerable evidence supports the notion that an altered balance between $Foxp3^+CD4^+$ T_{reg} cells and T effector cells in the intestinal microenvironment might contribute to the pathogenesis of IBD[12,15]. The ability to control inflammatory lesions with transferred T_{reg} cells has been demonstrated in several IBD models[16,17]. On the other hand, patients with IBD have been found to harbor significantly reduced numbers of peripheral T_{reg} cells or increased serum level of soluble IL-2Rα[18,19]. Therefore, it is possible that a novel therapy involving T_{reg} cells might effectively treat IBD.

This review will focus on the functions of T_{reg} cells in controlling peripheral immune tolerance within the context of mucosal immunity and will explain the molecular pathogenesis of IBD and possible therapeutic strategies involving T_{reg} cells.

PATHOGENESIS OF IBD

The functions of mucosal immune system depend on the presence of intestinal flora[20]. In the intestinal epithelium, four cell types are found, including columnar cells, goblet cells, endocrine cells, and leukocytes to rest on a continuous basement lamina[21]. In addition, the immune cells in the lamina propria (LP), the leukocytes also involve various immune cells, such as the unique immune cell types that exist in gut- or mucosa-associated lymphoid tissues such as Peyer's patches, mesenteric lymph nodes, and isolated lymphoid follicles. Balanced mucosal immunity in the gut is important for both immune homeostasis and defense[22-24]. Intestinal immunity is maintained by the proportions of immune cells such as dendritic cells (DCs), macrophages, effector $CD4^+$ or $CD8^+$ T cells, and T_{reg} cells.

Previously reported genome-wide association studies have identified more than 100 distinct loci that confer risk or protection from IBD development although a substantial proportion of these loci are common to both diseases[6]. Many IBD-associated pathways are known to exert heterogeneous effects upon activation in different cell types, and the combination of these cellular outcomes might affect disease manifestation[8,25]. IBD-associated loci can be broadly categorized into several critical pathways such as the innate immune response, intestinal barrier, microbial defense, reactive oxygen, and antimicrobial activity[26]. For example, in humans, nucleotide-binding oligomerization domain-containing protein 2 (NOD2), which is encoded by the *NOD2* gene on chromosome 16 plays an important role in the intestinal immune system where it recognizes bacterial peptides and stimulates immune reactions[27]. Mutations in *NOD2* gene have been associated with CD[27]. Furthermore, $NOD2^{-/-}$ mice are susceptible to and demonstrate excessive intestinal inflammation when compared with control mice[28]. Treatment with NOD2 ligands, including peptide or muramyl dipeptide has been shown to ameliorate 2,4,6-trinitrobenzenesulfonic acid (TNBS)- or dextran sulfate sodium (DSS)-driven colitis in normal mice[29,30]. Treatment with *Lactobacillus* peptidoglycan was shown to increase the number of $CD103^+$DCs and $Foxp3^+$ T_{reg} cell in mesenteric lymph nodes and IL-10 expression in the colonic mucosa in a TNBS-driven model of colitis, suggesting that NOD2 activity in the intestinal mucosa potentiates a tolerogenic environment[30].

Homing or migration-associated receptors such as CD62L, C-C chemokine receptor (CCR)7, αEβ7 integrin, α4β7 integrin, CCR4, CCR5, and CCR9 also contribute to the pathogenesis of IBD[31-37]. The expression of these receptors on T_{reg} cells plays a key role in the intestinal immunological homeostasis and defective expression of these receptors has been shown to induce IBD as a result of the deficient migration of T_{reg} cells into the intestine. For example, a loss of CCR7 was found to block T_{reg} cell function in an experimental model of colitis[32].

ANIMAL MODELS OF IBD AND T_{reg} CELLS

Many animal models of IBD have been developed based on the various aspects in the pathogenesis or mechanisms of IBD[16,38]. Mice have been widely used to examine the contribution of bacteria and specific bacterial factors to the pathogenesis of IBD[9]. Studies conducted in germ-free, specific pathogen-free, and gnotobiotic mice have suggested that defined or specific microbial flora plays a fundamental role in

the initiation and development of IBD[39-41]. Additional experimental colitis mouse models have been induced using chemical drugs such as DSS, TNBS, oxazolone, or acetic acid[9,42-46]. In addition, adoptive transfer models in which a T-cell deficient mouse strain is reconstituted with T_{reg} cell-depleted naïve T cells from a congenic donor mouse have been widely used[16,38,47]. Additional genetic models, including gene knockout, transgenic, or mutant animals, have been utilized to understand the genetic and molecular pathogenesis of IBD[9,16,38]. As spontaneous models of IBD, several animal models mice and rats are known to understand the pathogenesis of IBD or investigate new therapeutic strategy for IBD[9,38].

Bacteria-infected models

Studies conducted in mouse models of IBD have indicated that the T_{reg} cell compartment is particularly sensitive to microbiotal changes[48]. T_{reg} cells from germ-free mice are generally less suppressive and express lower level of Foxp3 than do T_{reg} cells from normal mice[49-51]. The colons of germ-free mice harbor reduced number of T_{reg} cells, although this population was shown to increase in response to a decrease in bacterial load following vancomycin treatment[52,53]. *Citrobactor rodentium*-infected mouse models have been the preferred infection models in acute intestinal inflammation[54]. Helicobacter-infected mice have been also used to be a bacterial infection model in combination with the other model[47]. In addition, toll-like receptor 9 (TLR9)-deficient mice harbor increased numbers of T_{reg} cells in the small intestine, suggesting that inflammatory signals from bacterial DNA play a role in inhibiting induced T_{reg} cell differentiation or T_{reg} cell proliferation[12,55].

Gene-manipulated models

IL-2$^{-/-}$, *IL-2R*$^{-/-}$, and *IL-10*$^{-/-}$ mice are known IBD models in which reduced T_{reg} cell number or dysfunction of T_{reg} cells are observed[41,19,56-60]. In addition, innate or mucosal immunity-related gene-deficient mice, such as *NOD2*$^{-/-}$, myeloid differentiation primary response 88 (*MYD88*)$^{-/-}$, nuclear factor-κB (*NF-κB*)$^{-/-}$, cytokine-deficiency induced colitis susceptibility 1 (*CDCS1*)$^{-/-}$, multidrug resistance gene 1a (*MDR1a*)$^{-/-}$, and *TLR5*$^{-/-}$ mice, also reveal inflammatory lesions in the colon[27,61-70]. T-cell receptor (*TCR*)$^{-/-}$ mice are also known to be one of IBD models[60]. Overexpression of *TNF-α* and signal transducer and activator of transcription4 (*STAT4)* gene in mice results in the development of IBD-like lesions[16,71]. Moreover, there are various gene-manipulated models, in which epithelial barrier- and immune regulation-associated genes are manipulated by knockdown, knockin, conditional knockout, or transgenic mice (Table 1)[9,16,38,39,72-82]. Among them, a reduction of T_{reg} cell number or impaired T_{reg} cell function is observed in several models (Table 1)[19,59,60,68,72,77,78].

Spontaneous models

As one of spontaneous IBD models based on the pathogenic factor by T_{reg} cells, senescence-accelerated mouse protein (SAMP)1/YitFc (SAMP) mice are well known[83]. A functional abnormality in T_{reg} cells in SAMP mice is observed[83]. C3H/HeJBir mice with high susceptibility to bacteria have been also used to be a model of IBD[84]. In addition, a monkey model, cotton-top tamarin, infected with *Campylobacter Jejuni* is known to be a spontaneous model of IBD[85]. Moreover, long-evans cinnamon (LEC) rat is a spontaneous IBD model based on T_{reg} cell-associated pathogenicity[86]. The LEC rat was first described as a naturally occurring mutant with a defect in thymocyte development: specifically, T-cell differentiation is arrested at the transition from $CD4^+CD8^+$ double positive to $CD4^+CD8^-$ single positive (SP), but not to $CD4^-CD8^+$ SP, thymocytes[87]. Accordingly, in LEC rats, peripheral $CD4^+$ T cells did not function as Th cells in terms of antibody production against T cell-dependent antigens, as well as in IL-2 production[87]. In addition, significantly reduced numbers of thymic and peripheral $Foxp3^+T_{reg}$ cell and a defect in the suppressive activity of T_{reg} cells were observed in these rats[86]. Interestingly, the proportion of T_{reg} cells in the LP is significantly decreased in LEC rats when compared with control rats[86]. These findings suggest that the dysfunction in the T_{reg} cell-controlled regulatory system may play a crucial role in the development of IBD.

REGULATORY T CELL

Differentiation of T_{reg} cells

Forkhead box P3 (Foxp3)-expressing T_{reg} cells are a suppressive subset of $CD4^+$ T cells that control autoimmunity, allergy, infection, and tumors[11,88]. Natural T_{reg} (nT_{reg}) cells arise as a discrete and largely stable lineage in the thymus[89,90]. The nT_{reg} subset exhibits a TCR repertoire that is distinct from those of $Foxp3^-$conventional T cells and induced (iT_{reg}) cells[91-94].

nT_{reg} cells require IL-2 for the development and maintenance and were initially identified by their elevated expression of the high-affinity IL-2 receptor (CD25)[11]. Mice that lack the ability to conduct IL-2-mediated signaling following the injection of anti-IL-2 mAb or as a result of *IL-2* or *IL-2R* gene knockout exhibit defects in the number and function of nT_{reg} cells[95-98]. The most important finding in mice with deficient IL-2 signaling is the development of spontaneous autoimmune lesions, including IBD[96-98].

nT_{reg} cells share phenotypic features with naïve and memory conventional T cells[89]. Most T_{reg} cells in circulation and the lymphoid organs express CCR7 and the adhesion receptor CD62L, which direct their recirculation through lymphoid tissues; effector or activated T_{reg} cells comprise a minor fraction of these[88,99]. Similar to activated conventional T cells, this T_{reg} cell population exhibits phenotypes

Table 1 Animal models of inflammatory bowel disease

Group	Model	Association	Ref.
Chemical drug-induced model	DSS	TNF-α, IL-17, reduced T_{reg} cells	[43]
	TNBS	IL-12, IL-17, reduced T_{reg} cells	[44]
	Oxazolone	IL-4	[45]
	Acetic acid (rat)	Myeloperoxidase	[46]
Adoptive transfer model	Naïve T cell→*Rag*$^{-/-}$/SCID	Recovery by T_{reg} cell transfer	[16]
Bacteria-infected model	*C. rodentium*	Acute infection, IL-17	[54]
	Helicobacter, *etc.*	Combined with the other models	[47]
Spontaneous model	SAMP/Yit/Fc	Dysfunction of T_{reg} cell	[83]
	C3H/HeJBir	Increased susceptibility to bacteria	[84]
	Cotton-top Tamarin (monkey)	*C.Jejuni* infection	[85]
	LEC (rat)	Reduced T_{reg} cells	[86]
Gene-manipulated model	*IL-2*$^{-/-}$	Reduced T_{reg} cells	[59]
	IL-2R$^{-/-}$	Reduced T_{reg} cells	[19]
	IL-10$^{-/-}$	Reduced T_{reg} cells	[60]
	NOD2$^{-/-}$	Impaired innate immunity	[61]
	Myd88$^{-/-}$	Impaired TLR signal	[65]
	NF-κB$^{-/-}$	Impaired pro-inflammatory signal	[62]
	Cdcs1$^{-/-}$	Impaired pro-inflammatory signal	[67]
	Mdr1a$^{-/-}$	Reduced iT_{reg} cell differentiation	[68]
	TCR$^{-/-}$	Defective adaptive immunity	[60]
	TGFβ$^{-/-}$	Reduced T_{reg} cell	[72]
	JAK3$^{-/-}$	Balance of Th1 and Th2	[73]
	Muc2$^{-/-}$	ER stress	[74]
	A20$^{-/-}$	Impaired Myd88 signal	[75]
	TCR$^{-/-}$*SOCS1*$^{-/-}$	Increased IFN-γ and IL-4	[76]
	CD4/*TGFβ*$^{-/-}$	Impaired T_{reg} cell function	[77]
	CD4/*PDK1*$^{-/-}$	Reduced T_{reg} cell	[78]
	CD4/*Blimp1*$^{-/-}$	Increased IL-17	[79]
	TNF$^{\Delta ARE}$TG	Increased CD8 function	[71]
	*STAT4*TG	Increased TNF-α	[80]
	T/*CD40L*TG	Thymic dysfunction	[81]
	B/*CD40L*TG	Increased IFN-γ	[82]

such as CD62L^{lo}CCR7^{lo}CD44^{hi} killer cell lectin-like receptor subfamily G member 1 (KLRG1)$^{+}$CD103^{+} or CD45RA^{lo}CD25^{hi} is thought to have encountered antigens[33,100-102]. These cells exhibit enhanced migration through nonlymphoid tissues.

Tissue-resident T_{reg} cells can be found in non-lymphoid tissues, even under noninflammatory conditions[89,103]. Various tissues such as the skin, lungs, liver, salivary gland, lacrimal gland, intestinal mucosa, adipose tissue, and placenta are known to harbor substantial numbers of T_{reg} cells in both humans and mice[14,104]. For example, CCR7^{+}Foxp3^{+} T_{reg} cells were found to reside in the salivary glands of healthy humans and mice whereas the number of CCR7^{+}Foxp3^{+} T_{reg} cells was extremely decreased in the tissues from patients and model mice with Sjögren's syndrome, an autoimmune disease that affects the exocrine glands such as the salivary and lacrimal glands[104,105].

Peripheral T_{reg} cells

iT_{reg} cells are generated from peripheral naïve conventional CD4^{+} T cells during the course of an immune response[12]. In healthy mice, most peripheral T_{reg} cells in the spleen and lymph nodes are thymus-derived nT_{reg} cells. By contrast, the iT_{reg} population resides in the intestinal LP and gut-associated lymphoid tissue (GALT)[48,106]. iT_{reg} cell generation involves naïve T-cell activation in the presence of transforming growth factor-β (TGF-β): in this setting, antigen exposure induces iT_{reg} cell differentiation[107]. iT_{reg} cells are particularly prevalent at mucosal surfaces where tolerance is induced against various antigens within the colonizing microbiota[52,108]. In addition, ingested food contains a plethora of antigens: in particular, food antigen feeding experiments identified a TGF-β1-producing CD4^{+} T cell population in the LP and GALT[109]. Immune responses to these dietary molecules can be actively suppressed by inducing oral tolerance[110]. Experiments with germ-free mice have suggested that bacterial commensals are essential for the development of normal numbers of colonic T_{reg} cells[109]. In the human microbiota, 17 clostridial strains that induce T_{reg} cell gut-homing and expansion by producing large amounts of short-chain fatty acids (SCFAs), which are bacterial breakdown products of plant-derived fibers were identified[111,112]. In particular, the SCFAs propionate, butyrate, and acetate can restore clonal T_{reg} cell numbers in germ-free or antibiotic-treated mice[112].

Although the gastrointestinal tract harbors the largest reservoir of tissue-resident T_{reg} cells in the

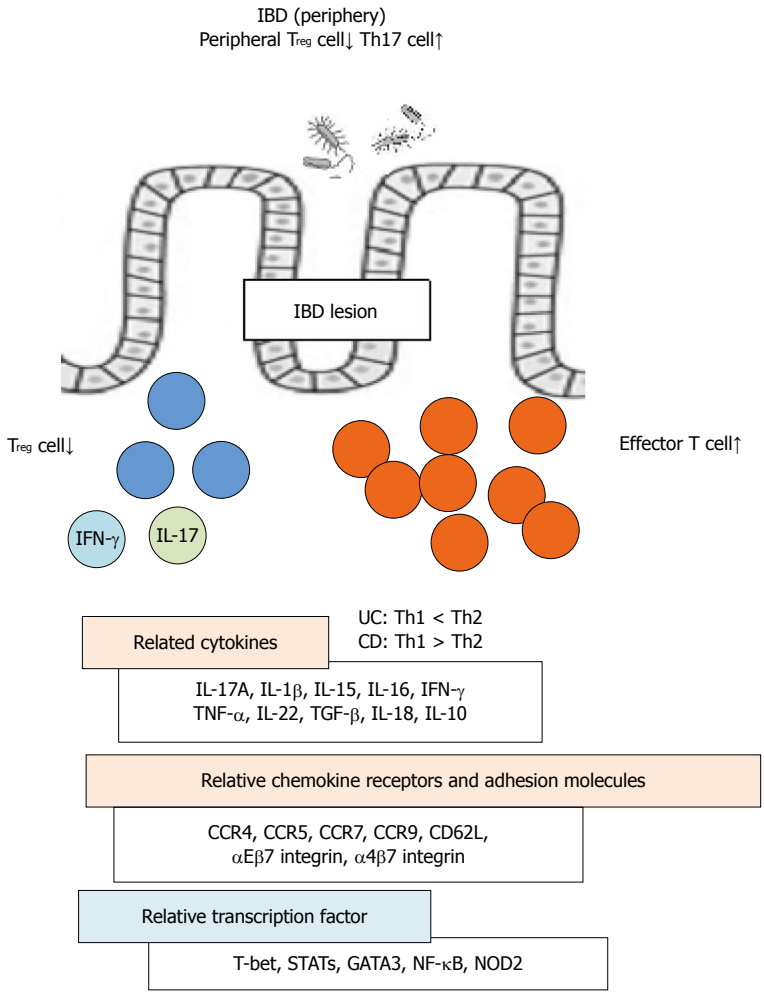

Figure 1 Molecular contribution to the pathogenesis of inflammatory bowel disease. In the lesions of inflammatory bowel disease (IBD), a various immune cells and epithelial cells contribute to the onset or development of the disease. Imbalance between cytokines or abnormal expression of chemokine receptors and adhesion molecules through several transcription factors in immune cells including effector T cell influences the pathogenesis of IBD. Decreased number of T_{reg} cells and abnormal differentiation of T_{reg} cells into effector-like phenotype are observed in the IBD lesions.

body, recent studies have demonstrated that T_{reg} cells are also highly enriched in the visceral adipose tissue (VAT)[113]. Interestingly, more than 50% T_{reg} cells of $CD4^+$ T cells in the VAT of 20-week-old mice are found[113]. Differences in the expressions of IL-10, CCR1, CCR2, CCR4, GATA binding protein 3 (GATA3), and peroxisome proliferator-activated receptor γ (PPARγ) have been observed between VAT T_{reg} cells and effector T_{reg} cells in lymphoid tissues[113,114]. Moreover, mice with a T_{reg} cell-specific *PPARγ* deletion that were fed a high-fat diet exhibited impaired restoration of insulin sensitivity and glucose tolerance following pioglitazone treatment[113,114]. These findings suggest that VAT T_{reg} cells might be critical mediators of the effects of treatment for lipid metabolism disorders.

REGULATOY T CELL IN IBD

Phenotypes of T_{reg} cells in IBD

The phenotype and function of T_{reg} cells in the inflamed mucosa or periphery of patients with IBD or animal models have been described as considerably different from those in peripheral lymphoid organs of healthy controls[12,115] (Figure 1). For example, patients with IBD exhibit reduced numbers of peripheral T_{reg} cells and increased numbers peripheral Th17 cells[116]. By contrast, the mRNA expression levels of Foxp3 are elevated in the mucosa of patients with IBD along with elevated levels of IL-17A, IL-1β, and IL-6 mRNA[116]. In IBD models, dysfunctional suppressive T_{reg} cell function plays a crucial role in the development of IBD lesions *via* the upregulation of T-cell specific T-box transcription factor (T-bet), STAT-1, and nuclear factor-κB (NF-κB)-mediated peripheral T_{reg} cell activation[86]. Intestinal immune system activation in response to bacterial antigens and pathologic cytokine production by intestinal T cells is induced through various transcription factors or signal molecules, including T-bet, GATA-3, and STATs and plays a critical role in the development of IBD[72,117-119]. CD is associated with the production of Th1 cytokines such as IFN-γ and TNF-α and in an IBD model naïve T cells transferred into T cell-deficient mice can be controlled by a coinjection of T_{reg} cells which suppress Th1 effector cell

functions such as IFN-γ production[120,121]. Moreover, UC development in humans is associated with Th2 cytokines such as IL-5[122].

Recent studies have demonstrated that $Foxp3^+$ T_{reg} cells express retinoic acid receptor-related orphan receptor gamma t and are thus able to differentiate into Th17 cells, a process that is associated with a decreased suppressive T_{reg} cell function in patients with IBD. T_{reg} cells were found to suppress colonic inflammation by downregulating Th17 responsiveness *via* TGF-β in an adoptive transfer mouse model of colitis[123,124]. Additionally, CCR7 was shown to regulate the intestinal Th1/Th17/T_{reg} cell balance during CD-like murine ileitis[125]. Furthermore, IFN-γ^+IL-17^+ coproducing $CD4^+$ T cells which express high levels of T-bet, CD26, and IL-22 resemble the pathogenic Th17 cells that contribute to intestinal inflammation in IBD[126]. Epithelial-derived IL-18 also regulates both colonic Th17 cell differentiation and T_{reg} cell function in the context of IBD-associated intestinal inflammation[124].

A recent study described *Clostridium* bacteria as potent inducers of $Foxp3^+$ T_{reg} cells in the colonic mucosa[111]. Additionally, $CD4^+CD8^+\alpha\alpha$ colonic T_{reg} cells were reported to produce IL-10 in response to *Faecalibacterium prausnitzii* and the frequencies of $CD4^+CD8^+\alpha\alpha$ lymphocytes in the LP lymphocytes and peripheral blood were found to be significantly lower in patients with IBD than in healthy controls[127]. These findings suggest that colonic bacteria-induced $CD4^+CD8^+\alpha\alpha$ T_{reg} cells may control or prevent IBD.

Therapeutic Treg cell-based strategies for IBD

New and effective therapies involving anti-TNF antibodies have yielded remarkable progress in the field of IBD therapy[10]. In addition, clinical trial applications are underway for target molecules such as IL-6/IL-6R, IL-12/23, IL-17A/F, IL-13, interferon-gamma-inducible protein 10 (IP10), CCR9, Janus kinase 3 (JAK3), similar to mothers against decapentaplegic (Smad)7/TGF, α4β7/β7, and mucosal vascular addressin cell adhesion molecule (MAdCAM)[4,5]. Target molecules such as IL-17A/F, IL-13, IP10, JAK3, α4β7/β7, or MAdCAM also represent promising therapeutic strategies for UC[4,5,15,22,24].

The $Foxp3^+T_{reg}$ cell frequency was found to be significantly lower in active patients with IBD than in healthy controls[18,116]. On day 14 after an initial dose of anti-TNFα infusion therapy, patients with IBD exhibited a significant increase in the frequency of circulating T_{reg} cells along with two- to three-fold increase in Foxp3 expression, which paralleled a reduction of IBD[128]. Furthermore, another report demonstrated that anti-TNFα mAb [infliximab (IFX)] therapy yielded a significant and sustained relative increase in peripheral blood T_{reg} cells; a change in C-reactive protein levels and durable clinical response was associated with this sustained increase in circulating $Foxp3^+T_{reg}$ cells[129]. IFX therapy was also shown to downregulate the mucosal expression of Foxp3 mRNA and protein in patients with UC and CD[129].

In patients with active IBD, anti-TNF-α treatment rapidly enhances the frequency of functional $Foxp3^+T_{reg}$ cells in the blood and potentiates their suppressive function[128]. In addition, in these patients increased apoptosis of local $Foxp3^+T_{reg}$ cells is observed in the inflamed mucosa when compared with noninflamed control colon tissues, along with a reduced frequency and increased apoptosis of peripheral blood T_{reg} cells and elevated caspase activity in the serum[130]. During anti-TNF-α antibody treatment, a decrease in the apoptosis of T_{reg} cells was found to correlate closely with an increase in peripheral T_{reg} cell numbers and a decrease of caspase and disease activity[130].

$CD4^+CD25^+CD127^{lo}CD45RA^+$ T_{reg} cells appear to be a promising population from which to expand T_{reg} cells for autologous T_{reg} cell transfer therapy in patients with CD[131]. Expanded $CD45RA^+$ T_{reg} cells carry an epigenetically stable *FOXP3* locus and do not convert to a Th17 phenotype in *in vitro* culture and $CD45RA^+$ T_{reg} cells from patients with CD were found to home to the human small intestine in a C.B-17 severe combined immune deficiency xenotransplant model[131]. There remains an unmet need for the development of novel therapies for IBD, as current drug therapies frequently fail to maintain long-term remission and may be complicated by significant side effects. Although cellular therapies are emerging as a potentially attractive many hindrances to successful clinical therapy remain in terms of sufficient cell numbers, cell maintenance, and cell sources.

CONCLUSION

Although the precise molecular pathogenesis of IBD remains unclear, recent therapeutic strategies involving antibodies against pathogenic cytokines or chemokines have provided treatment opportunities for many more patients suffering from IBD. Additionally, T_{reg} cells may provide a more effective therapy for IBD; therefore the unknown mechanisms with respect to T_{reg} cell differentiation, function, and maintenance should be clarified.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

miRNAs as new molecular insights into inflammatory bowel disease: Crucial regulators in autoimmunity and inflammation

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Abstract

Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammatory disorders of the gastrointestinal tract, and includes two major phenotypes: ulcerative colitis and Crohn's disease. The pathogenesis of IBD is not fully understood as of yet. It is believed that IBD results from complicated interactions between environmental factors, genetic predisposition, and immune disorders. miRNAs are a class of small non-coding RNAs that can regulate gene expression by targeting the 3'-untranslated region of specific mRNAs for degradation or translational inhibition. miRNAs are considered to play crucial regulatory roles in many biologic processes, such as immune cellular differentiation, proliferation, and apoptosis, and maintenance of immune homeostasis. Recently, aberrant expression of miRNAs was revealed to play an important role in autoimmune diseases, including IBD. In this review, we discuss the current understanding of how miRNAs regulate autoimmunity and inflammation by affecting the differentiation, maturation, and function of various immune cells. In particular, we focus on describing specific miRNA expression profiles in tissues and peripheral blood that may be associated with the pathogenesis of IBD. In addition, we summarize the opportunities for utilizing miRNAs as new biomarkers and as potential therapeutic targets in IBD.

Key words: Autoimmunity; Immune system; Inflammation; Inflammatory bowel disease; miRNA

Core tip: Inflammatory bowel disease (IBD) is characterized by chronic relapsing inflammation in the gastrointestinal tract, but its pathogenesis remains unclear. Further understanding of the molecular mechanisms of IBD is helpful to find new therapeutic strategies. miRNAs play crucial regulatory roles in immune cellular differentiation and maturation, and maintaining immune homeostasis. Aberrant expression of miRNAs is present in IBD. Here, we summarize how miRNAs regulate autoimmunity and inflammation, and describe specific miRNA expression profiles in IBD. We also discuss the opportunities in utilizing miRNAs as new biomarkers and potential therapeutic targets in IBD.

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises ulcerative colitis (UC) and Crohn's disease (CD). IBD is characterized by chronic relapsing inflammation in the gastrointestinal tract, and its incidence and prevalence are increasing[1]. The precise pathogenic mechanism of IBD remains unknown. Accumulated evidence suggests that IBD results from complicated interactions between environmental factors, genetic predisposition, and immune dysregulation. Of these, immune dysregulation is believed to play an important role in the pathogenesis of IBD[2]. Consequently, it is important to uncover the molecular mechanisms that regulate the immune responses in IBD.

miRNAs are a new class of small non-coding RNAs that regulate immune responses in physiologic and pathologic conditions[3]. miRNAs are considered to play significant roles in many biologic processes, including cellular proliferation, differentiation, maturation, and apoptosis[4]. In addition, miRNAs have been implicated in the pathogenesis of common human diseases, such as cardiovascular[5], neurologic[6], and hematologic diseases, cancer[7], and inflammatory and autoimmune diseases[8]. These research findings have led to new insights into IBD pathogenesis.

In this review, we summarize recent findings that miRNAs regulate autoimmunity and inflammation by affecting the differentiation, maturation, and function of various immune cells. We particularly focus on providing evidence of specific miRNA expression profiles in IBD pathogenesis. In addition, we also discuss the possibility for miRNAs as new biomarkers and potential therapeutic targets in IBD.

GENERAL OVERVIEW OF MIRNA

miRNAs are a new class of small (about 22 nucleotides), endogenous, non-coding single-stranded RNA molecules that can negatively regulate target gene expression at the post-transcriptional level[9]. The first miRNA, *lin-4*, was identified in 1993 in *Caenorhabditis elegans*[10]. The miRNA sequence database, miRBase, contains 35,828 mature miRNAs in 223 species at time of publication (http://www.mirbase.org/, Release 21, June 2014)[11].

miRNA genes are located either within intronic sequences of protein-coding genes, within intronic or exonic regions of non-coding RNAs, or within intergenic regions[12]. The biogenesis of miRNAs includes two parts: one is transcription in the nucleus, and the other is generation of mature miRNAs in the cytoplasm. First, miRNA is transcribed from the genome by RNA polymerase Ⅱ or Ⅲ to generate primary miRNA[13,14]. The primary miRNA is then cleaved by RNase Ⅲ-type enzyme Drosha to produce a pre-miRNA of approximately 70 nucleotides with a stem-loop structure in the nucleus[15]. Next, the pre-miRNA is exported to the cytoplasm by Exportin 5[16]. Once in the cytoplasm, the pre-miRNA is cleaved by Dicer in cooperation with protein partners, into an approximately 22-nucleotide miRNA duplex[17]. Then, one strand is selected as a functional miRNA, while the passenger strand is degraded. The functional miRNA is loaded into the RNA-induced silencing complex and acts as a guide strand that recognizes the target mRNA by complementary sequences[18]. Full complementarity occurs in plants, resulting in target mRNA degradation. However, incomplete complementary binding occurs in humans, and this leads to mRNA destabilization and translational inhibition[12].

MIRNAS AND THE INNATE IMMUNE SYSTEM

The innate immune system forms the first line of host defense, which is non-specific, and responds to pathogens in a generic way. It is comprised of tissue barriers, immune cells, and immune molecules. The tissue barriers include mechanical (epithelial) barriers, chemical barriers such as antimicrobial peptides, and biologic barriers (commensal flora). The innate immune cells include monocytes/macrophages, dendritic cells (DCs), neutrophils, natural killer (NK) cells, NK T cells, mast cells, eosinophils, and basophils. These cells perform phagocytosis, antigen presentation, and activation of the adaptive immune responses[19]. miRNAs regulate autoimmunity and inflammation by affecting the differentiation, maturation, and function

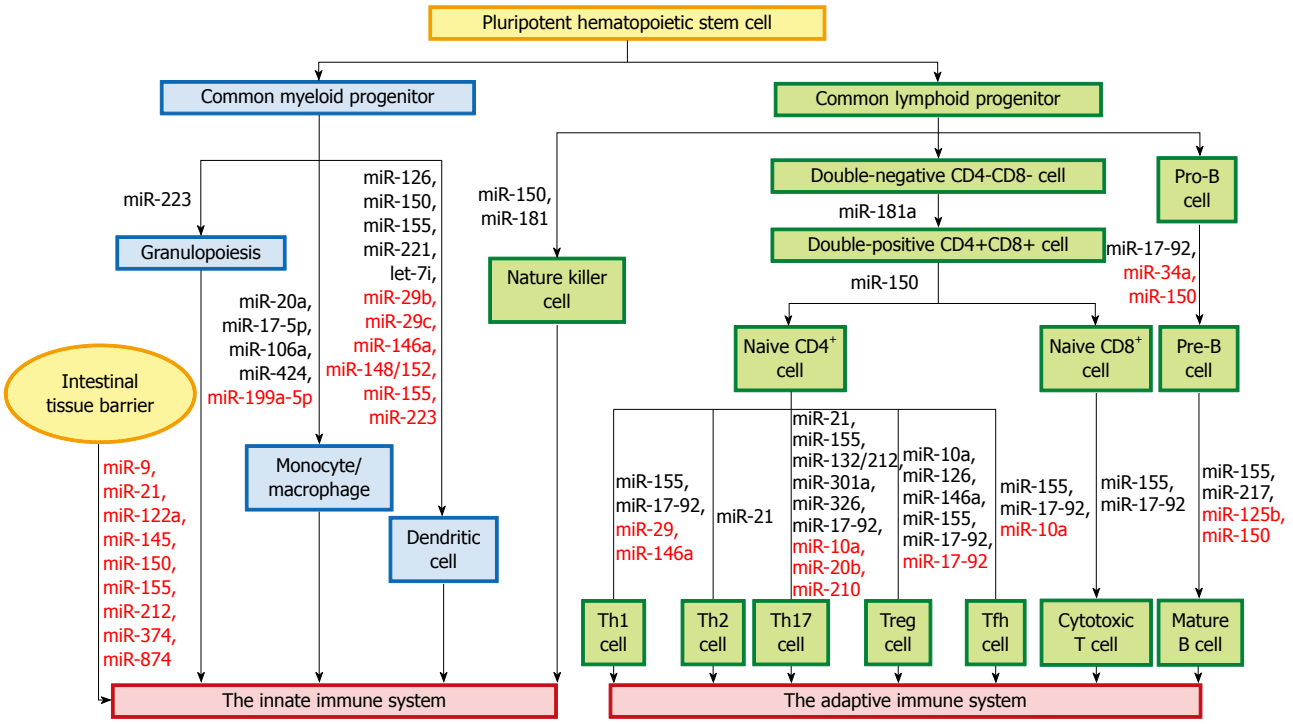

Figure 1 miRNAs and the immune system. miRNAs regulate autoimmunity and inflammation by affecting the differentiation, maturation, and function of various immune cells. The miRNAs in black letters are positive regulators in maintaining the differentiation and function of immune cells, while those in red letters act as negative regulators of these processes.

of various immune cells (Figure 1).

miRNAs and intestinal tissue barriers

The intestinal mucosa forms a barrier that separates luminal contents from the interstitium. Tissue barriers include tight junctions (TJs), adherens junctions, and desmosomes, which regulate the paracellular permeability of epithelial layers across the apical/basolateral axis[20]. The main protein complexes of TJs are composed of transmembrane proteins such as claudins, occludin and junctional adhesion molecules[21]. Disruption of the intestinal barrier has been shown to be an important pathogenic mechanism contributing to the development of intestinal inflammation[22,23]. McKenna *et al*[24] reported that miRNAs are important for maintaining the function of intestinal barriers.

miR-21, which is overexpressed in chronic UC, induces the degradation of Ras homolog gene family member (Rho)B mRNA and leads to an increase in intestinal epithelial permeability due to the loss of TJ proteins and ultrastructural changes[25]. miR-150 is significantly elevated in colon tissue in dextran-sulfate-sodium-induced murine experimental colitis and active UC patients. Overexpression of miR-150 results in intestinal epithelial disruption through targeting of c-Myb[26]. Both occludin and claudin-1 have been demonstrated to be involved in miR-874-induced intestinal barrier dysfunction by targeting the 3′-untranslated region of aquaporin 3[27]. miR-9 and miR-374 directly target the 3′-untranslated region of claudin-14 mRNA, leading to claudin-14 mRNA translational repression and decay, in a cooperative manner[28]. miR-145 impairs TJ function by repressing junctional adhesion molecule-1 expression[29]. miR-212 impairs the intestinal epithelial barrier by downregulating zonula occludens-1 protein expression, which is another major component of TJs[30].

Tumor necrosis factor (TNF)-α is an essential mediator of inflammation in the gut. Anti-TNF-α therapy induces remission in patients with severe active CD[31], UC[32], and refractory celiac disease[33]. TNF-α-induced upregulation of miR-122a mediates the degradation of occludin mRNA in enterocytes and influences their permeability[34]. TNF-α-induced miR-155 overexpression inhibits synthesis of zonula occludens-1 by downregulating RhoA expression[35].

miRNAs and monocytes/macrophages

Monocyte/macrophage differentiation is an essential branch of hematopoiesis, which is under the control of a complex network of regulatory factors[36]. During monocytopoiesis, the transcription factor acute myeloid leukemia (AML)1 is upregulated, while miRNAs-17-5p/20a/106a are downregulated. Monocytopoiesis is regulated by a circuitry comprising sequential miRNAs-17-5p/20a/106a, AML1, and monocyte colony-stimulating factor receptor, whereby miRNAs-17-5p/20a/106a act as a master gene complex that negatively regulates AML1 expression[37]. The transcription factor PU.1 upregulates miR-424 expression, and this induces monocyte differentiation *via* miR-424-dependent translational inhibition of nuclear factor (NF)I-A. This result indicates an important role of miR-424 and its target NFI-A in

controlling monocyte/macrophage differentiation[38]. miR-199a-5p targets the activin A receptor type 1B gene, leading to decreased expression of CCAAT/enhancer binding protein α, and eventually, inhibits monocyte/macrophage differentiation[36].

miRNAs and DCs

DCs serve as the most potent antigen-presenting cells, responsible for primary immune responses. Accumulating evidence highlights the importance of specific miRNAs in DC development, antigen-presentation capacity, and cytokine release[39]. miR-146a[40], miR-155[41], and let-7i[42] are involved in the maturation and functional state of DCs, while miR-148/152[43] and miR-223[44] are involved in their antigen-presentation capacity. miR-150 is required for the cross-presentation capacity of Langerhans cells (skin-resident DCs)[45]. In addition, miR-29b, miR-29c[46], miR-126[47], miR-146a[40,48], miR-155, and miR-221[49] have been shown to regulate DC apoptosis and cytokine production.

miRNAs and NK cells

NK cells are cytotoxic lymphocytes that play a vital role in host defense against infection, and they mediate antitumor responses. Recent advances have demonstrated that miRNAs are crucial in NK cell biology[50]. For instance, miR-150 and miR-181 regulate NK cell development[51,52]. Mice lacking miR-150 are defective in generating mature NK cells. On the contrary, transgenic mice with a gain-of-function miR-150 have enhanced NK cell development[51]. miR-181 promotes NK cell development by targeting Nemo-like kinase, which is a inhibitor of Notch signaling[52].

miRNAs and other kind of innate immune cells

Invariant NK T cells are a separate subset of T lymphocytes with innate effector functions. A Dicer-dependent miRNA pathway is important in the regulation of invariant NK T cell differentiation, function, and homeostasis[53]. The normal granulocytic differentiation requires the zinc finger protein growth factor independent-1, which is a transcription inhibitor that regulates the expression of miR-21 and miR-196b during myelopoiesis[54]. In addition, miR-223 plays a crucial role in the regulation of granulocyte differentiation and function, and mediates inflammatory responses[55,56].

miRNAs and activation of the innate immune system

Pattern recognition receptors are critical for the recognition of microorganisms and the induction of immune and inflammatory responses[57]. The families of these proteins include the membrane-bound Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene-I-like receptors[58]. Pattern recognition receptors promote downstream signaling cascades. Emerging evidence indicates that miRNAs regulate these processes.

TLRs: miR-146a expression can be induced through exposure to TLR ligands, such as lipopolysaccharide, peptidoglycan, and flagellin, and this induction is controlled by NF-κB. Mice lacking miR-146a are more likely to develop autoimmune diseases, tumorigenesis, and myeloid cell proliferation. miR-146 targets TNF-receptor-associated factor 6 and interleukin (IL)-1-receptor-associated kinase 1, which are key elements of the myeloid differentiation factor 88 pathway, and form a negative feedback mechanism in TLR signaling[59-61]. miR-155 expression is also induced by TLR signaling[61,62]. Unlike miR-146a, miR-155 promotes the immune response. Mice deficient in miR-155 are highly resistant to experimental autoimmune encephalomyelitis[63].

NOD-like receptors: Of this family of proteins, NOD2 functions as an intracellular sensor that contributes to inflammation and immune defense. It has been identified as the strongest single genetic locus in determining susceptibility in CD[64]. A miRs-NOD interaction has been implicated in IBD. These miRNAs include miR-29[65], miR-122[66], miR-146a[67], and miR-192[68]. For example, polymorphisms in NOD2 impair miR-29 expression in DCs, and this results in exaggerated IL-23-induced inflammation[65]. miR-122 targeting of NOD2 has a crucial role in the damage of intestinal epithelial cells induced by lipopolysaccharide[66].

MIRNAS AND THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system mainly consists of two different lymphocytes (T and B cells), and is highly pathogen specific. The appropriate development and function of these two immune cells are essential when distinguishing foreign from resident antigens. Current studies have indicated that miRNAs play important roles in maintaining the differentiation and function of T and B cells[69].

miRNAs and T-cell regulation

Increasing evidence shows that some specific miRNAs participate in the regulation of crucial immune functions. These immuno-miRs play significant roles in T-cell development, maturation, activation, differentiation, and aging[70]. For example, miR-150[71] and miR-181a[72] are involved in T-cell development, while miR-21[73] and miR-17-92 cluster[74,75] participate in T-cell activation.

miRNAs and T-helper 1/2 cell differentiation

miRNAs have significant effects on T helper (Th) cell

differentiation. Naïve T cells can differentiate into Th1, Th2, or Th17 cells after activation[76]. Th1 cells have been associated with the pathogenesis of CD, while Th2 cells have been implicated in UC[19]. miR-155 promotes Th1 differentiation by targeting interferon (IFN)-γ receptor α chain[77]. In contrast, $CD4^+$ T cells deficient in miR-155 display a bias towards Th2 differentiation, which is partly due to increased expression of the Th2-associated transcription factor c-Maf[41,78]. miR-17-92 cluster promotes Th1 differentiation. The function of miR-19b is mediated through phosphatase and tensin homolog, while miR-17 targets transforming growth factor (TGF)-β receptor Ⅱ and cAMP-responsive element-binding protein 1[79]. miR-29 regulates Th cell differentiation by directly targeting T-box transcription factor T-bet and eomesodermin to suppress IFN-γ production[80]. miR-146a may be a potent inhibitor of Th1 differentiation by targeting protein kinase Cε[81]. miR-21 promotes Th2 differentiation[82].

miRNAs and Th17 cell differentiation

Th17 cells, a new subset of Th cells capable of producing IL-17, play a vital role in the formation of several autoimmune-mediated inflammatory diseases, including IBD[83,84]. Current studies demonstrate that miR-21[85], miR-155[63], miR-301a[86], miR-326[87], miR-17-92 cluster[88], and miR-132/212 cluster[89] act as positive regulators of Th17 differentiation. For example, miR-155 enhances the development of inflammatory T cells (Th1 and Th17 cells), and facilitates Th17 cell formation through cytokines produced by DCs[63]. miR-10a[90], miR-20b[91], and miR-210[92] act as negative regulators of Th17 differentiation. Deletion of miR-210 promotes Th17 differentiation under hypoxic conditions[92].

miRNAs and regulatory T cells

Regulatory T (Treg) cells are another subset of $CD4^+$ T cells that can suppress activity of effector T cells and maintain self-tolerance[76,90]. Treg cells can be classified into two populations, naturally-occurring Treg (nTreg) cells that are generated in the thymus, and inducible Treg (iTreg) cells that arise from naïve $CD4^+$ precursors in the periphery[90]. miRNAs play pivotal roles in the regulation of both Treg cell development and function[93,94]. miR-10a is highly expressed in nTreg cells and can be induced by retinoic acid and TGF-β in iTreg cells[90]. Repression of miR-10a *in vitro* results in reduced forkhead box (Fox)p3 expression levels, while ablation of miR-10a does not affect the phenotype or number of nTreg cells[93]. miR-155-deficient mice display a marked reduction in the number of Treg cells. Additionally, miR-155 maintains homeostasis of Treg cells by targeting suppressor of cytokine signaling 1 *via* the IL-2 signaling pathway[95]. miR-146a is important for maintaining suppressive function of Treg cells. Treg cells deficient in miR-146a lead to immunologic intolerance by targeting signal transducer and activator of transcription-1[96]. Silencing of miR-126 can influence the expression of Foxp3 on Treg cells and impair their suppressive function *via* the PI3K/Akt pathway[97]. miR-17-92 cluster is also involved in Treg cell function. Mice with Treg-specific loss of miR-17-92 cluster develop an exacerbated form of experimental autoimmune encephalomyelitis and fail to achieve clinical remission[74]. However, there are conflicting results. For instance, the study from Jiang *et al*[79] showed that miR-17-92 cluster prevents Treg cell differentiation and promotes Th1 responses.

miRNAs and follicular helper T cells

Follicular helper T (Tfh) cells are a novel subset of $CD4^+$ T cells that can provide help to B cells, and they are important for germinal center formation[98]. Several studies have demonstrated that miRNAs are crucial for Tfh cell differentiation and function[99,100]. Mice with T-cell-specific loss of miR-17-92 cluster exhibit severely compromised Tfh cell differentiation, germinal center formation, and antibody responses. On the contrary, T-cell-specific miR-17-92 cluster transgenic mice spontaneously accumulate Tfh cells[99]. miR-10a attenuates phenotypic conversion of iTreg cells to Tfh cells by simultaneously targeting Bcl-6, a transcription factor critical for Tfh cell differentiation[101], along with the corepressor Ncor2[90]. Moreover, miR-155 has been reported to promote Tfh cell development[102].

miRNAs and $CD8^+$ T cells

$CD8^+$ T cells or cytotoxic T lymphocytes can devastate various intracellular pathogens and malignancies[103]. Dicer is required for $CD8^+$ T-cell survival and accumulation, but not required for the early steps in $CD8^+$ T-cell activation[104,105]. Dicer and miRNAs such as miR-139 and miR-150 also participate in controlling the cytolytic program, as well as other programs of effector cytotoxic T lymphocyte differentiation[106]. miR-155 is demanded for effector $CD8^+$ T-cell responses to viral and intracellular bacterial infection and cancer. miR-155 has the potential to be a target for immunotherapy for infectious diseases and cancer[103,107,108]. miR-17-92 cluster has dynamic regulation of $CD8^+$ T cells differentiating from naïve to effector and memory states[75,109].

miRNAs and B cells

Ablation of Dicer in early B-cell progenitors leads to a formative block from the pro-B to pre-B transition[110]. miRNAs are also involved in B-cell development and function[110,111], including miR-34a[112], miR-150[113,114], and miR-17-92 cluster[115]. miR-125b inhibits B-cell differentiation in germinal centers[116]. In addition to regulating B-cell differentiation, miR-150[113], miR-155[78], and miR-217[111] regulate B-cell function, including the establishment of B-cell tolerance, as well as antigen-dependent and -independent antibody repertoire diversification.

Table 1 Aberrantly expressed miRNAs in inflammatory bowel disease

Sample type	Expression	miRNAs	Ref.
Ulcerative colitis *vs* healthy controls			
Mucosal tissues	Upregulated	miR-7, miR-16, miR-20b, miR-21, miR-23a, miR-24, miR-29a, miR-29b, miR-31, miR-125b-1*, miR-126, miR-126*, miR-127-3p, miR-135b, miR-146a, miR-150, miR-155, miR-195, miR-206, miR-223, miR-324-3p, miR-424, and let-7f	[25,26,117-122]
	Downregulated	miR-188-5p, miR-192, miR-200b, miR-215, miR-320a, miR-346, miR-375, and miR-422b; miR-124 (pediatric cases)	[117,119,123,124]
Peripheral blood	Upregulated	miR-16, miR-21, miR-28-5p, miR-103-2*, miR-151-5p, miR-155, miR-188-5p, miR-199a-5p, miR-340*, miR-362-3p, miR-378, miR-422a, miR-500, miR-501-5p, miR-532-3p, miR-769-5p, miR-874, and miRplus-E1271	[125-127]
	Downregulated	miR-505*	[126]
Crohn's disease *vs* healthy controls			
Mucosal tissues	Upregulated	miR-9, miR-21, miR-22, miR-26a, miR-29b, miR-29c, miR-30b, miR-31, miR-34c-5p, miR-106a, miR-106b, miR-126, miR-126*, miR-127-3p, miR-130a, miR-133b, miR-146a, miR-146b-5p, miR-150, miR-155, miR-181c, miR-196a, miR-196, miR-206, miR-324-3p, miR-375, and miR-424	[119,121,130,131]
	Downregulated	miR-7 and miR-141	[128,129]
Peripheral blood	Upregulated	miR-16, miR-23a, miR-29a, miR-106a, miR-107, miR-126, miR-191, miR-199a-5p, miR-200c, miR-362-3p and miR-532-3p; miR-16, miR-20a, miR-21, miR-30e, miR-93, miR-106a, miR-140, miR-192, miR-195, miR-484, and let-7b (pediatric cases)	[125,133]

MIRNAS AND IBD

Abnormal miRNA expressions exist in some diseases, including IBD (Table 1). This offers a new way to improve our comprehension of the mechanism of this disease. Moreover, some specific miRNAs in IBD may serve as potential biomarkers for diagnosis, evaluation indicators of disease activity, or targets for treatment.

miRNAs in UC

miRNAs in mucosal tissues: In 2008, the first profiling study of altered expression of miRNAs in IBD patients was published[117]. Wu and colleagues[117] found a specific miRNA expression pattern: three miRNAs (miR-192, miR-375, and miR-422b) were markedly downregulated, whereas eight miRNAs (miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, and let-7f) were observably upregulated in active UC tissues. Furthermore, they found that miR-192 participated in the regulation of chemokine production in colonic epithelial cells. Since 2008, some new research in active UC and healthy controls has confirmed the upregulation of miR-21[25,118], miR-29a[119], and miR-126[120], and identified additional upregulated miRNAs, including miR-7, miR-29b, miR-126*, miR-127-3p, miR-135b, miR-223 and miR-324-3p[119], miR-31[119,121], miR-150[26], miR-155[118], miR-146a, miR-206, and miR-424[121], and miR-20b and miR-125b-1*[122]. In contrast, miR-188-5p, miR-215, miR-320a, miR-346[119], and miR-200b[123] were downregulated in colon tissues from active UC patients compared with healthy controls. miR-124 was markedly decreased in pediatric but not in adult UC tissues. Reduced levels of miR-124 in colon tissues appear to increase the expression and activity of signal transducer and activator of transcription-3, and this mediates the pathogenesis of UC in children[124].

miRNAs in peripheral blood: Paraskevi and colleagues[125] found that six miRNAs (miR-16, miR-21, miR-28-5p, miR-151-5p, miR-155, and miR-199a-5p) were remarkably upregulated in blood from UC patients compared with healthy controls. miR-155 had the highest expression level of these six UC-associated miRNAs in peripheral blood. Wu and colleagues[126] found that compared with healthy controls, 12 miRNAs were significantly upregulated, and miRNA-505* was downregulated in blood from active UC patients. Peripheral blood miRNAs may distinguish active UC patients from healthy controls. As compared to active CD patients, ten miRNAs were markedly upregulated, and one miRNA was downregulated in blood from active UC patients[126]. Duttagupta *et al*[127] completed analyses of miRNA expressions from different hematologic fractions as noninvasive predictors for incidence of UC. They found that seven miRNAs derived from platelets (miR-188-5p, miR-378, miR-422a, miR-500, miR-501-5p, miR-769-5p, and miR-874) were upregulated. This study provides new platelet-derived miRNA biomarkers for clinical application and perception of the potential roles of these miRNAs in the pathogenesis of UC.

miRNAs in CD

miRNAs in mucosal tissues: Most studies of miRNA expression profiles in CD have concentrated on Crohn's colitis. Fasseu *et al*[119] found that 23 miRNAs (miR-9, miR-21, miR-22, miR-26a, miR-29b, miR-29c, miR-30b, miR-31, miR-34c-5p, miR-106a, miR-126, miR-126*, miR-127-3p, miR-130a, miR-133b, miR-146a, miR-146b-5p, miR-150, miR-155, miR-181c, miR-196a, miR-324-3p, and miR-375) were remarkably upregulated in colonic tissues from CD patients compared with healthy controls. Five of these miRNAs were specific for patients in

an active stage of CD (miR-9, miR-126, miR-130a, miR-181c, and miR-375), whereas the remaining 18 were also upregulated in colonic tissues from inactive CD patients. Huang and colleagues[128] identified that miR-141 was downregulated in inflamed colon tissues from active CD patients. miR-141 inhibited colonic chemokine CXC ligand 12β expression by directly targeting it and blocked colonic immune cell recruitment. Nguyen and colleagues[129] found that only miR-7 was downregulated in eight active colonic CD patients compared to six healthy controls. In addition, miR-206, miR-424[121], miR-106b[130] and miR-196[131] are also upregulated in active colonic CD.

However, is there any tissue-specific miRNA expression profile in the gastrointestinal tract? Wu and colleagues[132] examined miRNA expression patterns in tissues from different intestinal segments in active CD patients. Ten intestine-specific miRNAs (miR-19b, miR-22, miR-23a, miR-26a, miR-31, miR-126, miR-215, miR-320, miR-422b, and let-7d) were identified. Specifically, three of these (miR-22, miR-31, and miR-215) were markedly upregulated in the terminal ileum compared with colon tissue, while miR-19b was downregulated in the terminal ileum. Moreover, miR-23a, miR-26a, miR-126, miR-320, miR-422b, and let-7d showed colon-specific expression. In active colonic CD patients, three miRNAs (miR-23b, miR-106, and miR-191) were upregulated and two (miR-19b and miR-629) were downregulated compared to healthy controls. In active terminal ileal CD patients, four miRNAs (miR-16, miR-21, miR-223, and miR-594) were upregulated in terminal ileal tissues.

miRNAs in peripheral blood: Apart from assessing miRNA expressions in peripheral blood in UC, Paraskevi *et al*[125] examined miRNA expression patterns in peripheral blood samples from 128 patients with active CD and 162 healthy individuals. Eleven miRNAs (miR-16, miR-23a, miR-29a, miR-106a, miR-107, miR-126, miR-191, miR-199a-5p, miR-200c, miR-362-3p, and miR-532-3p) were markedly upregulated in peripheral blood from CD patients as compared with healthy individuals. There were no significant differences in miRNA expressions in accordance with disease location and phenotype.

Zahm *et al*[133] examined serum samples from 46 pediatric CD patients and 32 healthy controls by means of a low-density microarray and quantitative reverse transcriptase (qRT) PCR. They found 11 miRNAs (miR-16, miR-20a, miR-21, miR-30e, miR-93, miR-106a, miR-140, miR-192, miR-195, miR-484, and let-7b) that were CD-associated circulating miRNAs. Receiver operating characteristic analyses indicated that these CD-associated miRNAs had promising diagnostic value, with sensitivities of 70%-83% and specificities of 75%-100%. These results demonstrate that circulating miRNAs may be used as novel noninvasive biomarkers in CD.

miRNAs in IBD at different stages

Iborra and colleagues[134] assessed miRNA expression patterns in serum and tissue samples from nine patients with active UC, nine with inactive UC, nine with active CD, and nine with inactive CD, and serum from 33 healthy subjects. They found that two miRNAs (miR-548a-3p and miR-650) were higher, and three (miR-196b, miR-489, and miR-630) were lower in the mucosa of active UC patients compared with inactive UC patients. There were no differences in serum miRNA expression profiles in patients with active UC compared with inactive UC. However, there were differences in serum miRNA expressions between active and inactive CD patients; two serum miRNAs (miR-188-5p and miR-877) were increased, and four serum miRNAs (miR-18a, miR-128, miR-140-5p, and miR-145) were decreased in patients with active CD. Furthermore, four miRNAs (miR-18a*, miR-140-3p, miR-629*, and let-7b) were higher, and three miRNAs (miR-328, miR-422a, and miR-855-5p) were lower in the mucosa of active CD patients compared with inactive CD patients. These results indicate that there are specific miRNA expression patterns associated with different stages of IBD. Further prospective cohort studies in large samples are necessary to validate these findings.

miRNAs as therapy in IBD

miRNA-related therapeutic applications may represent a new and fascinating field in IBD treatment. miRNA-related therapy is based on antisense technology and gene therapy; thus, it involves either miRNA antagonists or miRNA mimics.

miRNA antagonists: miRNA antagonists include anti-miRNA oligonucleotides (AMOs), miRNA sponges, and miRNA masks.

AMOs: AMOs are synthetic anti-miRNA oligonucleotides with reverse complementary sequences to their target miRNAs, which suppress miRNA functions. It is believed that AMOs have a promising future in therapeutic applications. Chemical modifications of AMOs can improve their stability and binding affinity. Common modifications include addition of different 2′-ribose modifications to AMOs (2′-O-methyl and 2′-O-methoxyethyl) and 2′,4′-methylene bridge-locked nucleic acid (LNA). LNA-modified AMOs create high-affinity binding to target mRNAs[135,136]. A study by Janssen *et al*[137] demonstrated that miravirsen, an LNA-anti-miR-122, is designed to target and inhibit miR-122, and this can reduce viral RNA levels in patients with chronic hepatitis C virus infection. This result proves the possibility of miRNA agents in clinical practice.

miRNA sponges: miRNA sponge technology utilizes plasmid or viral vectors to achieve loss-of-function of miRNAs. The strong promoters can be applied in miRNA sponge vectors for generating high-level expression of the competitive inhibitor transcripts for either transient or long-term inhibition of miRNA function. Considering the merit of sharing a common seed sequence by members of a miRNA family, this technology provides a strong approach for coinstantaneous inhibition of multiple miRNAs of interest with a single inhibitor[138].

miRNA mimicry/replacement therapy: In order to restore miRNA activity, miRNA mimics (synthetic oligonucleotides) and miRNA expression gene vectors are used. MRX34 is a double-stranded miRNA mimic of the naturally occurring miR-34a loaded in liposomal nanoparticles to reestablish its tumor suppressor function. MRX34 was the first miRNA mimic introduced into clinical study for primary as well as metastatic liver cancer in 2013[139,140]. Many miRNAs are down-regulated in UC and CD. For example, miR-192, miR-375, miR-422b[117], miR-188-5p, miR-215, miR-320a, miR-346[119], and miR-200b[123] are decreased in UC, and miR-19b and miR-629[132] are decreased in Crohn's colitis tissues. Theoretically, replenishing these decreased miRNAs by miRNA mimics may provide therapeutic restoration of physiologic functions lost in IBD.

CONCLUSION

In this review, we described the roles of miRNAs as crucial regulators of inflammatory responses and autoimmune disorders, particularly focusing on miRNAs affecting the differentiation, maturation, and function of various immune cells. We also summarized some studies on the current understanding of the connection between miRNAs and IBD. Accumulating evidence suggests that specific miRNA expression profiles exist in IBD, and these miRNAs contribute to the development of inflammation. The definite functions of most miRNAs in IBD have not yet been clarified. Further studies are necessary to validate whether miRNAs could be used to diagnose IBD, distinguish IBD subtypes, and determine the disease activity or location.

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TOPIC HIGHLIGHT

2016 Irritable Bowel Syndrome: Global view

Gut microbiota role in irritable bowel syndrome: New therapeutic strategies

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Abstract

In the last decade the impressive expansion of our knowledge of the vast microbial community that resides in the human intestine, the gut microbiota, has provided support to the concept that a disturbed intestinal ecology might promote development and maintenance of symptoms in irritable bowel syndrome (IBS). As a correlate, manipulation of gut microbiota represents a new strategy for the treatment of this multifactorial disease. A number of attempts have been made to modulate the gut bacterial composition, following the idea that expansion of bacterial species considered as beneficial (*Lactobacilli* and *Bifidobacteria*) associated with the reduction of those considered harmful (*Clostridium*, *Escherichia coli*, *Salmonella*, *Shigella* and *Pseudomonas*) should attenuate IBS symptoms. In this conceptual framework, probiotics appear an attractive option in terms of both efficacy and safety, while prebiotics, synbiotics and antibiotics still need confirmation. Fecal transplant is an old treatment translated from the cure of intestinal infective pathologies that has recently gained a new life as therapeutic option for those patients with a disturbed gut ecosystem, but data on IBS are scanty and randomized, placebo-controlled studies are required.

Key words: Irritable bowel syndrome; Gut microbiota; Probiotics; Prebiotics; Synbiotics; Antibiotics; Fecal transplantation

Core tip: In the last decade, the gut microbiota has provided support to the concept that a disturbed intestinal ecology could promote development and maintenance of symptoms in irritable bowel syndrome (IBS). As a correlate, manipulation of gut microbiota represents a new strategy for the treatment of this multifactorial disease. Probiotics appear an attractive option in terms of both efficacy and safety, while prebiotics, synbiotics and antibiotics still need for-

mation. Fecal transplant has recently gained a new life as therapeutic option for those patients with an altered gut ecosystem, but data on IBS are scanty and randomized, placebo-controlled studies are required.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a disorder characterized by chronic abdominal pain and discomfort associated with alterations of bowel habits in the absence of a demonstrable pathology[1,2]. Other common symptoms are abdominal distension, bloating and flatulence, straining and urgency. IBS is a common gastrointestinal (GI) disorders in the industrialized world with a 10%-15% prevalence in the general population[3]. This high prevalence together with the associated co-morbidities has a significant impact on both patients and society, especially in terms of quality of life and medical costs[4].

IBS is a heterogeneous functional disorder that, depending on the prevailing bowel habit, has been subtyped into IBS with constipation (C-IBS), IBS with diarrhea (D-IBS), mixed, alternate IBS (A-IBS) with both constipation and diarrhea plus unsubtyped IBS with neither constipation nor diarrhea[1,2]. Alterations of bowel habits are likely related to dysregulation of the autonomic system in the gut, whereas symptoms of abdominal pain and discomfort are thought to involve additional changes in the bidirectional communication between the gut and the brain, known as "gut-brain axis", that cause a modified perception of visceral events in the form of hyperalgesia or allodynia[5,6]. The etiology of IBS is incompletely understood and evidence is growing that IBS might be a post-inflammatory and stress-correlated condition[7,8]. Both host and environmental factors, including diet, play a key role in triggering symptoms. Among the host factors, central alterations (*i.e.*, aberrant stress responses, psychiatric co-morbidity and cognitive dysfunctions) and peripheral alterations (*i.e.*, intestinal dysmotility, visceral hypersensitivity, low-grade immune activation and altered intestinal barrier function) are both involved[9]. Despite considerable research efforts, the treatment of IBS remains a significant challenge mainly due to its poorly defined pathophysiology.

HUMAN MICROBIOTA

Human microbiota is a complex living ecosystem consisting of unicellular microbes, mainly bacterial, but also metagenomic archaeal (*i.e.*, *Methanobrevibacter*), viral (*i.e.*, bacteriophages) and eukaryotic (*i.e.*, yeast), that occupies almost every mucosal and cutaneous surfaces of our body. It has been estimated that microbes that stably live in human body amount to 100 trillion cells, ten-fold the number of human cells[10] and the majority inhabits the gut where the intestinal microbiota is widely regarded as a virtual organ that actively influences and mediates several physiological functions. These living microorganisms encode for over three millions of genes, the so-called "microbiome"[11], outfitting the human genoma by approximately 100-fold[12]. The intestinal microbiota is composed by 17 families, 50 genera and more than 1000 species of bacteria: its composition varies among individuals, changes during life and depends on environmental factors, mainly lifestyle, diet, drugs, stress and invasive medical procedures. The intestinal microbiota is dominated by four main phyla: *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria*. In the adult life *Bacteroidetes* and *Firmicutes* are usually prevalent, whereas *Actinobacteria* and *Proteobacteria* are less represented. In the healthy status, the gut microbiota interacts with the human host in a mutualistic relationship, the host intestine provides the bacteria with an environment to grow and the bacterial ecosystem contributes to maintain homeostasis within the host by modulating several physiological functions such as gut development[13], nutrient processing and digestion[14,15], immune cell development and immune responses[16-18], resistance to pathogens[19,20], control of host energy and lipid metabolism[14,15] and brain development and function. Changes in bacterial number and composition, the so-called dysbiosis, may induce a dysregulation of this deep relationship and cause the appearance of a spectrum of diseases including metabolic syndrome, diabetes, cancer, inflammatory conditions, neurological pathologies and psychiatric disorders.

Throughout its communication with gastrointestinal epithelial, immune and nerve cells, the gut microbiota generates and releases molecules that can signal to distant organs. It is now recognized that a significant portion of the metabolites circulating in mammalian blood derives from the intestinal microbial community[21-25] and the presence or absence of the gut microbiota influences the metabolic profile in regions distant from the gut such as the brain[26]. Moreover, it releases factors that target specific neuronal systems involved in the gut-brain axis, generating neurotransmitters and neuromodulators as dopamine, noradrenaline, acetylcholine and gamma-aminobutyric acid (GABA)[27-31]. Direct contact of certain probiotics (*i.e.*, *Lactobacillus acidophilus*) with epithelial cells induce the expression of opioid and cannabinoid receptors in the gut and contribute to the modulation and restoration of the normal perception of visceral pain[32]. Finally, as the result

of intestinal microbial colonization, metabolism, and subsequent fermentation, the human microbiota produces a significant proportion of the gases present in the gut, including carbon dioxide (CO_2), hydrogen (H_2), methane (CH_4), and hydrogen sulfide (H_2S). Since H_2S has been recently recognized as a gaseous neuromodulator/neurotransmitter capable of modulating intestinal inflammation and sensitivity[33-37], it may be hypothesized that the intestinal microbiota plays a significant role in modulating visceral pain also by producing this gaseous mediator. The term microbiota-gut-brain axis is now currently used to indicate the deep correlation among these three functional "organs".

MICROBIOTA AND IBS

IBS can be considered a multifactorial syndrome in which several pathogenic mechanisms are involved. Gut microbiota interferes with normal intestinal functions at diverse levels, acting as both cause and target of abnormalities of intestinal motility, sensitivity and neuroimmune signaling, including alterations of mucosal barrier and pattern recognition receptors expression, as well as dysfunctions of hypothalamus-pituitary-adrenal (HPA) axis.

Perturbation in microbiota composition

In recent years, perturbations in the intestinal microbiota have being linked to the pathophysiology of IBS (Figure 1), thought that studies investigating the composition of intestinal microbiota in IBS have produced non univocal results[38]. Nevertheless the majority of data support the notion that the composition of luminal and mucosal microbiota differs among specific subgroups of IBS patients and healthy individuals[39-63] (Table 1). Using culture-based techniques and a 16S rRNA gene-based phylogenetic microarray analysis, it has been demonstrated that the diversity of microbial population is reduced, the proportion of specific bacterial groups is altered and the degree of variability in the microbiota composition is different in IBS patients when compared with healthy subjects. Furthermore, a higher degree of temporal instability of the microbiota among IBS patients has been detected. Examples of these modifications are a decreased amount of *Lactobacilli* and *Bifidobacteria* along with an increased amount of aerobes relatively to anaerobes in IBS patients. Finally mucosal bacteria have also been found to be more abundant in IBS patients than in healthy controls (Table 1). Consistent with this view, the clinical guidance regarding the modulation of intestinal microbiota in IBS provided by the Rome Team Working Group has recently concluded that there is good evidence supporting the concept that the intestinal microbiota is perturbed in patients with IBS[64].

A part of the abnormal composition of gut microbiota in IBS, others factors support the notion that intestinal flora plays a key role in determining IBS. First, there is increasing evidence of an activation of the intestinal immune system in IBS leading in a micro-inflammation, with studies demonstrating increased concentrations of mucosal intraepithelial lymphocytes[65,66], mast cells[66-69] and 5-hydroxytryptamine-secreting enterochromaffin cells[65]. Gut microbiota influences mucosal inflammation in inflammatory bowel disease (IBD) patients, *i.e.*, ulcerative colitis (UC) and Crohn's disease. Animal-based studies emphasize the critical role of gut microbiota in the balance between immunosuppression and inflammation in the GI tract, involving Toll-Like Receptor (TLRs) signaling pathways[70]. Indeed, helmintic based treatment with *Heligmosomoides polygyrus* could ameliorate colonic inflammation in murine model of IBDs, shifting the composition of intestinal bacteria[71]. Furthermore, Kuehbacher *et al*[72] analyzed the gut microbiota of 73 patients with IBD demonstrating that alteration of TM7 bacteria and the genetically determined antibiotic resistance of TM7 species in these patients, could be a relevant part of a more general alteration of bacterial microbiota in IBD patients, *i.e.*, as a promoter of inflammation at early stages[72]. Recent studies demonstrate that reductions in protective bacteria and increases in inflammatory bacteria are associated with pouch inflammation in patients with UC who underwent pouch surgery[73]. Moreover, the presence of *Ruminococcus gnavus*, *Bacteroides vulgatus* and *Clostridium perfringens* and the absence of *Blautia* and *Roseburia* in faecal samples of patients with UC before surgery is associated with a higher risk of pouchitis after ileal pouch-anal anastomosis[74]. Given the evidence for the role of intestinal microbiota in the profound inflammatory state in IBD, it might be speculated that luminal antigens should play a similar role in development of subclinical inflammation in IBS. Second, it has been reported that approximately 10% of IBS patients refer that their symptoms began following an episode of infectious diarrhea[75-77], the so-called post infectious IBS (PI-IBS), a condition with a clear infective trigger that may alter the normal intestinal microbiota. Third, there is a strong association between IBS and prior use of antibiotics[78]. Fourth, the intestinal microbiota strongly interacts with exogenous factors, in particular diet, which may directly or indirectly cause IBS symptoms[79]. Fifth, it is well known that alteration of gut microbiota could interfere with behavior and mood in humans[80,81]; on the other hand, psychiatric disorders such as anxiety and depression are highly present as co-morbidities in individuals with IBS[82]. An high *Firmicutes*: *Bacteroides* ratio is found in some IBS patients and appears to correlate with depression and anxiety[60], while in another study it has been reported that in IBS patients with clinically significant anxiety, daily treatment with a prebiotic galactooligosaccharide

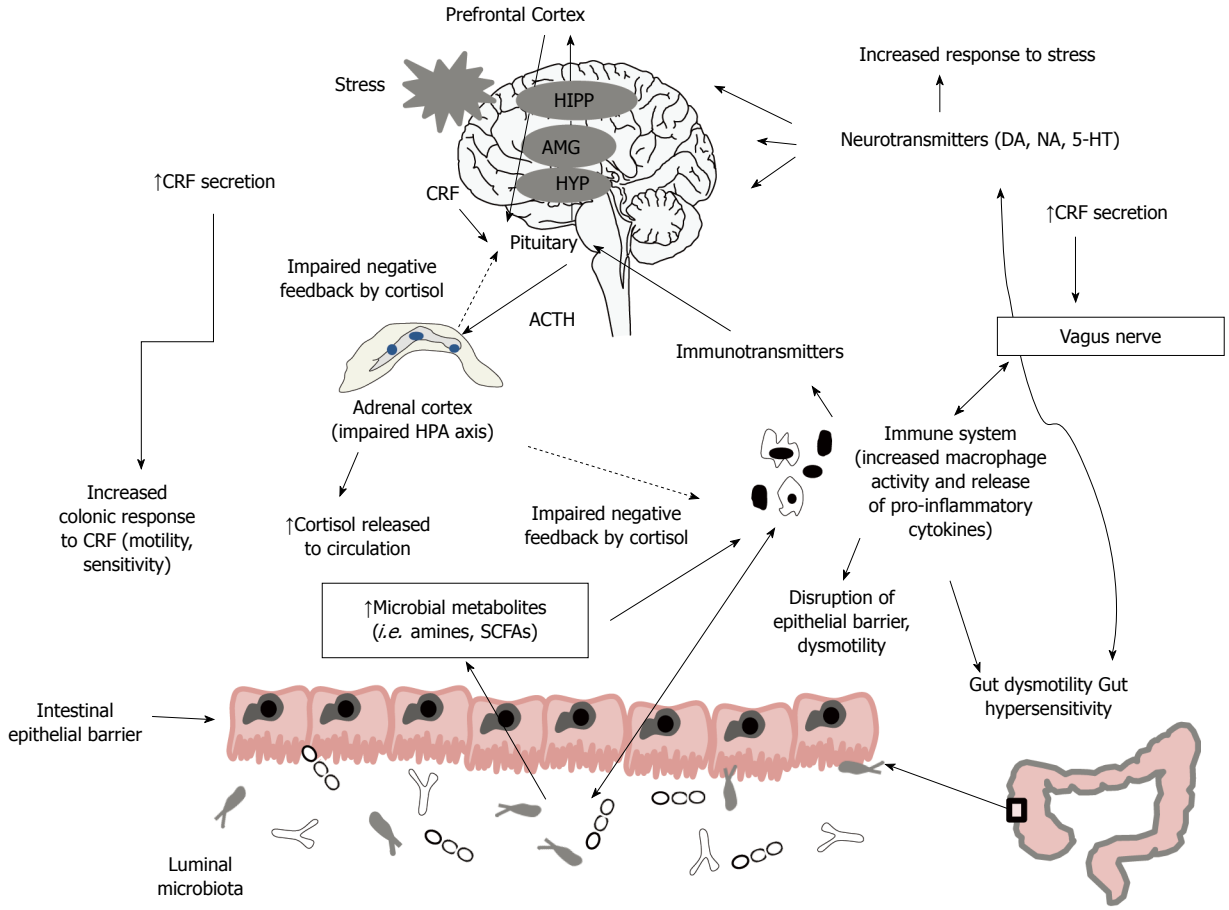

Figure 1 Gut microbiota influences the bidirectional communication between the enteric nervous system and the central nervous system, modulating gut development and several physiological functions, including intestinal motility, sensitivity, secretion and immunity. In irritable bowel syndrome (IBS), the altered composition and/or activity of microbiota may induce a disruption of this communication leading to activation of immune system and production of pro-inflammatory cytokines, production of microbial metabolites as short-chain fatty acids (SCFAs) that are toxic at high concentration, activation of hypothalamic-pituitary-adrenal (HPA) axis with increase of cortisol that feeds back to the pituitary, hypothalamus (HYP), amygdala (AMG), hippocampus (HIPP) and prefrontal cortex to shut off the HPA axis and increase of corticotropin releasing factor (CRF). These effects lead to alterations of intestinal motility and sensation, disruption of epithelial barrier and impaired production of neurotransmitters with an increased response to stressful events. On turn, stress may provoke systemic pro-inflammatory cytokines production that activates the HPA axis that signals to both enteric nervous system and the central nervous system and may alter microbiota composition.

mixture for 4 wk reduced anxiety scores and had a significant positive impact on quality of life[83]. Taken together, these factors support the notion that an imbalance in the intestinal microbiota composition may directly or indirectly interfere with the normal function of the microbiota-gut-brain axis, leading to the development of central and peripheral abnormalities of either intestinal motility and viscero-sensory network.

Microbiota and colo-intestinal motility

Although in the past decades the alterations of intestinal and colonic motility have been considered to play a major role in the development of symptoms in IBS patients[84-86], their influence has been reduced over time as intestinal manometry failed to identify any diagnostic abnormality in IBS patients[87,88]. The abnormal manometric findings found in IBS patients are heterogeneous and range from minimal changes to severe qualitative abnormalities. For example, the incidence of "clustered" contractions is similar in healthy subjects and IBS patients and greatly varies overtime[89,90], acute psychologic stress alters duodeno-jejunal motility in both healthy subjects and IBS patients[91] and more than half of IBS patients has an entirely normal 24-h manometry[92]. Nonetheless gut microbiota modulates gut motor function, which in turn can alter the intestinal microbiota composition. Indeed, not only germ-free animals show profound altered motility patterns that is reversed upon reconstitution with normal flora[93,94], but the influence of the intestinal microbiota on small intestinal myoelectric activity is species-dependent[95,96]. The modulatory effects of microbiota on colo-intestinal motility may be dependent on interaction of bacteria with the gastrointestinal tract through receptors on the epithelial cell such as TLRs and nucleotide oligomerization domain (NOD) receptors and, although bacterial translocation, defined as passage of viable bacteria to mesenteric lymph nodes or other organs, is minimal[97], secreted products of bacteria normally gain access to the submucosa to stimulate the mucosal immune system and to induce changes in intestinal

Table 1 Perturbations in the intestinal microbiotain irritable bowel syndrome patients

Ref.	Methods	N° of Pts.	Diagnostic criteria	Results in IBS in comparison with healthy subjects
Dlugosz *et al*[38]	qPCR (small bowel)	35	Rome Ⅱ	No differences
Balsari *et al*[39]				↓*Coliforms*
				↓*Lactobacilli*
				↓ *Bifidobacteria*
Si *et al*[40]	Culture	25	Rome Ⅱ	↓*Bifidobacteria*
				↑*Enterobacteriaceae*
Malinen *et al*[41]	qPCR	27	Rome Ⅱ	↑ *Veillonellain* C-IBS
				↓*Lactobacillus* in D-IBS
Mättö *et al*[42]	Culture/DGGE	26	Rome Ⅱ	↑ Aerobes
				Temporal instability
Swidsinski *et al*[43]	FISH	20	A-IBS	↑ Mucosal bacteria
			C-IBS	↑*Eubacteriumrectale*
			D-IBS	↑*Clostridium coccoides*
Maukonen *et al*[44]	PCR-DGGE	16	A-IBS	Higher instability of the bacterial population
			C-IBS	↑Clostridial groups
			D-IBS	
Kassinen *et al*[45]	16S ribosomal RNA gene cloning	24	Rome Ⅱ	Significant differences in several bacterial genera belonging to the genera *Coprococcus*, *Collinsella* and *Coprobacillus*
Lyra *et al*[46]	16S ribosomal RNA gene cloning	20	A-IBS	↑*Clostridium thermosuccinogenes* in D-IBS
			C-IBS	↑*Ruminococcus torques* in D-IBS
			D-IBS	↑*Ruminococcus bromii*-like in C-IBS
Krogius-Kurikka *et al*[47]	16S ribosomal RNA gene cloning	10	Rome Ⅱ	↑*Proteobacteria* and *Firmicutes* in D-IBS
				↓*Actinobacteria* and *Bacteroidetes* in D-IBS
Kerckhoffs *et al*[48]	FISH, PCR	41	A-IBS	↓*Bifidobacteria*
			C-IBS	
			D-IBS	
Carroll *et al*[49]	16S ribosomal RNA gene cloning	2	Rome Ⅲ	↑*Bacteroidetes*
				↑*Proteobacteria*
Salonen *et al*[50]				
Review				
Tana *et al*[51]	Culture	26	Rome Ⅱ	↑*Lactobacillus*
	qPCR			↑ *Veillonella*
Carroll *et al*[52]	qPCR	10	Rome Ⅲ	↓Aerobicbacteria
			D-IBS	↑*Lactobacillus*
Codling *et al*[53]	16S rRNA-DGGE	47	Rome Ⅱ	↓Bacterial richness
Ponnusamy *et al*[54]	rRNA-specific 16S rRNA-DGGE	54	Rome Ⅱ	Same total bacterial quantity
				Higher diversity of *Bacteroidetes* and *Lactobacilli*
	PCR			Lower diversity of *Bifidobacter* and *Clostridium coccoides*
Kerckhoffs *et al*[55]	16S rRNA-DGGE	37	Rome Ⅱ	↑*Pseudomonas aeruginosa*
	qPCR			
Rajilić-Stojanović *et al*[56]	16S rRNA	62	Rome Ⅱ	↑Ratio of the *Firmicutes* to *Bacteroidetes*
	qPCR			↑*Dorea*, *Ruminococcus*, *Clostridium spp*
				↓*Bacteroidetes*, *Bifidobacterium*, *Faecalibacterium spp*
Carroll *et al*[57]	16S rRNA	16	Rome Ⅲ D-IBS	Lower biodiversity of microbes
Carroll *et al*[58]	16S rRNA	23	D-IBS	↓Bacterial richness
				↑*Enterobacteriaceae*
				↑*Proteobacteria*
				↓*Faecalibacterium*
Parkes *et al*[59]	FISH	47	Rome Ⅲ	More total bacteria numbers
				↑*Bacteroides*, *Clostridia coccoides-Eubacteriumrectale*
Jeffery *et al*[60]	16S rRNA	37	Rome Ⅱ	A sub-group of IBS showed normal-like microbiota
				A sub-group of IBS showed large microbiota-wide changes with ↑*Firmicutes* and ↓*Bacteroidetes*
Chassard *et al*[61]	FISH/16S rDNA	14	Rome Ⅱ	↑*Enterobacteriaceae*
	Functional approaches		Rome Ⅲ	↑Suphate-reducing bacteria
			C-IBS	↓Lactic acid bacteria population (*bifidobacteria* and to a lesser extent, *lactobacilli*)
König *et al*[62]				
Review				
Sundin *et al*[63]	16s rRNA	19	Rome Ⅲ	↑*Bacteroidetes* in the PI-IBS group (13 patients)
				↑*Firmicutes* (more specifically *Clostridium* in IBS)

IBS: Irritable bowel syndrome; IBS-D: Diarrhea predominant IBS; IBS-C: Constipation predominant IBS; A-IBS: Alternate IBS; PI-IBS: Post-infectious-IBS; DGGE: Denaturing gradient gel electrophoresis; PCR: Polymerase chain reaction; FISH: Fluorescence in situ hybridization.

immunity and physiology. In this view, specific bacteria have been reported to induce significant changes in colo-intestinal motility. *Bacteroides thetaiotaomicron*, a common gut commensal in mice and humans, was found to alter expression of genes involved in smooth-muscle function and neurotransmission[98], soluble factors from the probiotic *Escherichia coli* Nissle 1917 enhance colonic contractility by direct stimulation of smooth muscle cells[99] and lipopolysaccharide (LPS) from a pathogenic strain of *Escherichia coli* impairs colonic muscle cell contractility[100]. Finally, exposure of human colonic muscle cells to *Lactobacillus rhamnosus GG* resulted in a significant dose- and time-dependent impairment of acetylcholine-stimulated contraction[101] and in the restoration of the intrinsic myogenic response in a model of LPS-induced alterations of muscle cells[102].

Microbiota and visceral sensitivity

The connections among gut and brain involve several integrated structures that transport the sensorial information from peripheral (gut) to the central (CNS) stations. Each stimulus from splanchnic visceral afferences (*i.e.*, distensive, chemical, thermal, osmotic) pass throughout the gut intrinsic innervation enteric nervous system (ENS), is received in the spinal dorsal horn and is transmitted to supraspinal sites, the final integration of the painful perception occurring in the cortex[103]. These complex communications connect to the extrinsic innervation [the autonomic nervous system (ANS)] which interacts with the HPA axis affecting the visceral sensory motor functions[104]. Vagal afferences activation plays a modulatory role on the spinal visceral pain pathway[105]. Visceral hypersensitivity may develop at several levels of the brain-gut axis, *i.e.*, ENS, spinal cord and supraspinal sites[106] and plays a key role in the pathogenesis of IBS, the main physiopathological alteration being represented by a reduced pain threshold to rectal distension[6,107,108]. Moreover, an altered rectal compliance[109,110] and/or an increased sensorial colonic response to intestinal lipid perfusion may be present[110-112]. An abnormal central processing of intestinal stimuli could be the cause of visceral hypersensitivity, as indicated by brain imaging studies that have shown an altered vascularization of certain areas of the CNS in response to intestinal distension in IBS patients, such as the anterior cingulated cortex, the amygdale and the dorso-medial frontal cortex[113]. At supraspinal sites, interactions with emotional or stressful stimuli can modulate the visceral sensitivity resulting to increased pain perception[114]. Recent data have shown that gut microbiota may directly modulate several systems involved in visceral hypersensitivity. Indeed, antibiotics-induced intestinal dysbiosis modified colonic pain-related and motor responses by upregulation of TLR4 and TLR7 and downregulation of the antinociceptive cannabinoid 1 and mu-opioid receptor in mice[115]. Moreover, manipulations of the commensal microbiota by stressful events was able to enhance the local expression of visceral sensory-related systems within the colon, as cannabinoid receptor type 2 and tryptophan hydroxylase isoform 1 (TPH1), leading to an excitatory modulation of visceral sensitivity[116]. Functional dysbiosis caused visceral hypersensitivity in patients affected by IBS (including PI-IBS), SIBO and chronic constipation by acting on local or systemic immune activation and altered intestinal fermentation[117], and gut commensals modulate the activations of intestinal sensory endings[118]. Probiotic strains directly modulate visceral perception of nociceptive stimuli. For example, *Lactobacillus reuteri* inhibited the autonomic response to colorectal distension in rats through effects on enteric nerves[119], modulated vagal afferents[120] and decreased the *in vitro* and *in vivo* activation of the transient receptor potential vanilloid 1 (TRPV1) channel which activity may mediate nociceptive signals[121].

Microbiota and autonomic nervous system, hypothalamic-pituitary-adrenal axis, enteric nervous system, mucosal barrier and neuro-immune signalling

The bidirectional communication network among central and peripheral regions includes the CNS, the spinal cord, the ANS, the ENS and the HPA axis. The autonomic system, *via* the sympathetic and parasympathetic branches, drives both afferent and efferent signals, while the HPA axis modulates the adaptive responses of the organism to stressors of any kind[122,123]. Stressful events, as well as elevated systemic pro-inflammatory cytokines, activate this system that, through secretion of the corticotropin-releasing factor (CRF) from the hypothalamus, stimulates adrenocorticotropic hormone secretion from pituitary gland that, in turn, leads to cortisol (an hormone that has a predominantly anti-inflammatory role on the systemic and GI immune system) release from the adrenal glands. Both neural and hormonal responses induce activation of several effector cells including immune cells, epithelial cells, enteric neurons, smooth muscle cells, interstitial cells of Cajal and enterochromaffin cells. Once activated, these systems exert a profound influence on gut microbiota composition both indirectly by modulating several GI functions (including motility, secretions, maintenance of intestinal permeability and integrity of immune response) and directly *via* signaling molecules[124]. On the other hand, these systems are under the influence of the gut microbiota composition that interacts not only locally with intestinal cells and ENS, but also directly with CNS through neuroendocrine and metabolic pathways[125-130].

Microbiota and pattern recognition receptors

The balance of innate signaling in the intestine is crucial to homeostasis and microbiota integrity is

essential in maintaining the neuro-immune function in the GI tract. The detection of pathogens by the host is obtained through the families of pattern recognition receptors (PRRs) that recognize conserved molecular structures known as pathogen-associated molecular patterns and induce production of innate effector molecules. These signaling receptors can be divided into three families: TLRs, retinoic acid inducible gene I-like receptors, and NOD-like receptors. The TLR family is the best characterized, and 13 receptors have been reported in mice and humans[131]. These PRRs play a key role in detecting pathogens and inducing the innate response. In particular, TLRs respond to specific microbial ligands and to harmful signals produced by the host during infection, initiating a downstream cascade that activates both innate and adaptive immunity. This cascade includes epithelial cell proliferation, secretion of IgA into the gut lumen and production of α-defensins, β-defensins and other bactericidal substances termed antimicrobial peptides (AMPs)[132-134]. Gut microbiota, through PRRs, can modulate the expression of genes involved in inflammatory and pain responses and the production of AMPs. In turn, the expression of PRRs affects the structure of gut microbiota in both health and disease. For example, alterations in the composition of the commensal microbiota as seen in dysbiosis may induce profound change in TLR4 and TLR7 expression, leading in alteration of colonic motility and sensitivity[115], while microbiota protects against ischemia/reperfusion-induced intestinal injury through NOD2 signaling[135]. In turn, TLR signaling maintains segregation between bacteria and the epithelium through production of AMPs[136-138], but deficiency in PRRs such as NOD2 and TLR5 can alter the gut microbiota composition in mice[139].

MODULATION OF THE INTESTINAL MICROBIOTA IN IBS

Probiotics

The term "probiotic" as originally defined by FAO/WHO refers to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[140]. However, in order to be beneficial, probiotic bacteria must be able to survive along the gastrointestinal tract, to resist to gastric acid, bile and pancreatic juice action and to demonstrate functional efficacy[141]. Several clinical trials that have investigated the therapeutic benefits of probiotics on either overall or specific IBS symptoms, but these studies are highly heterogeneous. Thus, although a number of meta-analysis or systemic reviews indicate that probiotics may be helpful in the treatment of IBS symptoms, their conclusions vary because of inadequate sample size, poor study design and use of various probiotic strains in the reviewed studies. This review has been focused on a number of meta-analysis and extensive reviews, published in the last 5 years, that have screened randomized and controlled trials (RCTs) conducted on IBS patients by using different probiotic strains.

Moayyedi *et al*[142] have examined 18 RCTs including 1650 patients with IBS and, although there was a significant heterogeneity among studies, a preference toward probiotic treatment was detected. Indeed, probiotic administration was significantly better than placebo at improving overall symptoms. No major difference was apparent between various types of probiotics used, with *Lactobacillus* (three trials, 140 patients)[143-145], *Bifidobacterium* (two trials, 422 patients)[146,147], *Streptococcus* (one trial, 54 patients)[148] and combinations of probiotics (four trials, 319 patients)[149-152], all showing a trend towards benefit, with no side effects reported. Moreover probiotics showed a statistically significant effect in improving individual symptoms such as pain, flatulence and bloating, but not urgency.

Lactic acid bacteria (LAB)[153], the most commonly used bacteria in probiotic preparations that include both typical and atypical species, covering *Lactobacilli, Bifidobacteria, Enterococci* and *Streptococci* have also been widely used in clinical trials. Analysis of 42 RCTs by Clarke *et al*[153] indicates that, despite a significant studies heterogeneity, 34 studies reported beneficial effects on at least one pre-specified endpoints or symptoms. Indeed, 20 of the 34 trials involving LAB reported improvement in abdominal pain/discomfort, 12 of the 24 trials reported improvement on abdominal bloating/distension, and benefits over placebo were reported in 13 of the 24 trials assessed using an index of defecatory function. Both *Bifidobacteria*[147,154-157] and *Lactobacilli*[144,145,154,158-165] were found effective in ameliorating IBS symptoms, while the beneficial effects of the multispecies LAB preparations, including the multistrain preparation VSL#3, were less evident[149,150,152,166-171].

A more strictly selected list of 16 RCTs was evaluated by Brenner *et al*[172]. These Authors found that 11 trials were inadequately blinded, of too short duration, of too small sample size, and/or lacked intention to treat analysis; they concluded that only two of the studies - those using *Bifidobacterium infantis 35624*[147-154] - showed significant improvements in abdominal pain/discomfort, bloating/distension and/or bowel movements, compared to the placebo. However, none of the studies provided quantifiable data about both tolerability and adverse events.

A systematic review by Hungin *et al*[173] has selected 19 studies and included 1807 patients. The majority of these studies tended to include all IBS subtypes, with only two studies focusing on C-IBS[156,157], and three studies focusing on D-IBS[149,174,175]. Although reported trials were extremely different for probiotic strains (above all *Lactobacilli* and *Bifidobacteria,* but also *Streptococcus salivaris*, *Saccharomyces boulardii*

and probiotic mixtures such as VSL#3), study design and definition of treatment response with a responder rates of 18%-80% in IBS group and 5%-50% in healthy controls, this review[149,156,157,173-188] detected several positive effects of probiotics on IBS symptoms and health-related quality of life. Probiotics had a favorable safety profile with no difference in adverse events among the 23 specific probiotic treatments and placebo[173].

Another systematic review with meta-analysis has been recently published by Didari *et al*[189]. They retrieved 11748 publication on probiotics, but only 15 were used for the meta-analysis, 9 of which were reviewed in deep. Again, the majority of the study was excluded for poor clinical design, lack of inclusion criteria, time limitation, and lack of a control group and use of probiotics in combination with herbal medications or prebiotics. The 15 trials used for the meta-analysis included 882 patients with D-IBS, C-IBS and A-IBS according to Rome Ⅱ, Rome Ⅲ, International Classification of Health Problems in Primary Care and World Organization of Family Doctors criteria[145,152,155,166,169,170,174-177,179,190-193]. Although the studies differ in term of bacterial strain used, probiotic dosage, duration of either treatment or follow-up and endpoints/outcome, probiotics were more effective than placebo in reducing abdominal pain after 8 and 10 wk of treatment; the effect was higher at week 8, suggesting a reduced effectiveness with long-term use. Furthermore, probiotics administration improved general IBS symptoms and the severity of symptoms was decreased, although not significantly in comparison with placebo. Few adverse events were reported in both probiotics and placebo groups. The same results have been reached by the extensive review of other 9 studies that, according to Rome Ⅱ or Rome Ⅲ criteria, included 324 patients with C-IBS, D-IBS and A-IBS[156,161,167,182,194-198].

As several trials have demonstrated the superiority of probiotics over placebo in controlling IBS symptoms (above all, *Lactobacilli* and *Bifidobacteria,* but also other species including bacterial mixtures such as VSL#3), there is now a general agreement on their efficacy as a therapy for this elusive syndrome (extensively discussed in Reference 112 and Table 2). However, given the controversies in IBS pathophysiology, patient heterogeneity, or lack of clear and reproducible evidence for gut microbiota abnormalities in patients with IBS, additional RCTs with appropriate end points and design are needed for determining to which extent (and in which IBS subpopulations) probiotics are a useful therapeutic strategy in the management of IBS symptoms.

Putative mechanisms of action of probiotics

As the pathogenesis of IBS is multifactorial, probiotics have been shown effective in modulating several mechanisms that might have a mechanistic role in IBS pathogenesis, including effects on composition of intestinal microbiota, gastrointestinal dysmotility, visceral hypersensitivity, altered gut epithelium and immune function, luminal metabolism, dysfunction of gut-brain axis, psychological distress.

Composition of intestinal microbiota in IBS

Only few trials on IBS patients have examined the composition of intestinal bacteria before and after the supplementation therapy, therefore the effect (if any) of probiotics administration on resident microbiota is not fully understood. However, it has been suggested that probiotics might reshape the intestinal eco-system generating an ecological milieu that is unfavorable for the growth of harmful species by increasing the number of *Lactobacilli* and *Bifidobacteria*[199] that will stabilize the intestinal microbiota[174,179]. As bacteria compete for nutrients and produce substances that directly affect the growth of other bacteria, probiotics can provide a two-fold protection against a broad range of pathogens, including certain forms of *Clostridium, Escherichia coli*, *Salmonella*, *Shigella* and *Pseudomonas*: aside from competing for the nutrients, probiotics also produce metabolites (*i.e.*, lactic acid, short chain fatty acids and hydrogen peroxide) and soluble factors (*i.e.*, bacteriocins as sakacin, lactocin, amylovorin, acidophilin, bifidin, bifidocin) that are inhibitory for some pathogenic bacteria[200,201]. Moreover, probiotics reduce the adherence of pathogenic bacteria by promoting the production of mucins[202-204].

Gastrointestinal dysmotility

From almost 4 decades, the assumption that IBS is characterized by impaired intestinal motor functions[205-207] and by gas retention[208] has driven the treatment of IBS to small bowel and colonic dysmotilities. In this conceptual framework probiotics are thought to directly affect the intestinal motility. Indeed, *Bifidobacterium Lactis* HN019 and *Bifidobacterium lactis* DN-173 010 decreased intestinal transit time in adult constipated patients[209] and a recent meta-analysis of randomized controlled trials have revealed that *Bifidobacterium lactis*, but not for *Lactobacillus casei* Shirota, reduced whole gut transit time and increased stool frequency in constipated patients[210]. Moreover, fermented dairy product containing *Bifidobacterium lactis* DN-173 010 reduced distension in association with acceleration of gastrointestinal transit and improvement of symptoms in IBS with constipation[156], daily *Bifidobacterium lactis* supplementation decreases WGTT and the frequency of functional GI symptoms in a dose-dependent manner in subjects suffering from irregular bowel movements and flatulence[185,211] and a combination of probiotics (*Bacillus subtilis* and *Streptococcus faecium*) was effective for relief of symptoms in patients with non-diarrheal-type IBS[212]. Probiotics are usefull also on D-IBS, as a probiotic mixture containg *Lactobacillus*

Table 2 Efficacy of probiotics in irritable bowel syndrome[112]

Authors' statement	Parameter scored	Conclusion	Grade of evidence for effect	Ref.
1	Global symptom assessment	Specific probiotics help relieve overall symptom burden in some patients with IBS	High	[147,149,150,152,156-157,169, 174,176-178]
2	Global symptom assessment	Specific probiotics help relieve overall symptom burden in some patients with C-IBS	Low	[147,156-157]
3	Global symptom assessment	Specific probiotics help relieve overall symptom burden in some patients with D-IBS	Moderate	[147,149,174-175]
4	Abdominal Pain	Specific probiotics help reduce abdominal pain in some patients with IBS	High	[145,147,149-150,152,155-157, 168-169,174-119]
5	Bloating/ distension	Specific probiotics help reduce bloating/distension in some patients with IBS	Moderate	[147,149-150,155-156,168-170, 174-177,179-183]
6	Flatus	Probiotics tested to date do not help reduce flatus in patients with IBS	Low	[147,149-150,156,168,174-175, 178-179,184]
7	Constipation	Specific probiotics may help reduce constipation in some patients with IBS	Low	[155-156,183,185]
8	Bowel habit	Specific probiotics help improve frequency and/or consistency of bowel movements in some patients with IBS	Moderate	[145,147,149-150,152,155-157, 168-170,174-180,182,185-186,188-189]
9	Diarrhoea	Probiotics tested to date do not reduce diarrhea in patients with IBS	Very low	[174,179,181-183,185,187]
12	Health-related quality of life	With specific probiotics, improvement of symptoms has been shown to lead to improvement in some aspects of health-related quality of life	Moderate	[147,150,152,155,169-170,174,176-177,179-180,183-184,186]

IBS: Irritable bowel syndrome; D-IBS: Diarrhea predominant IBS; C-IBS: Constipation predominant IBS.

acidophilus, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Bifidobacterium breve*, *Bifidobacterium lactis*, *Bifidobacterium longum*, and *Streptococcus thermophilus* has shown to be effective in controlling symptoms[174], but the effect on intestinal motility and transit time in this subtype of patients is less proven[149].

Visceral hypersensitivity

Mechanistic data provided mainly by animal studies highlight that probiotics exert a direct anti-nociceptive action through the modulation of bacterial metabolites production (*i.e.*, neurotransmitters, neuroactive substances including GABA and serotonin) on sensitive nerve endings in the gut mucosa[26-31,130], or by targeting specific central neurosensitive pathways. For instance, various strains of probiotics have been shown effective in reducing visceral nociceptive reflex responses in several experimental models of IBS[213,214] by directly modulating a number of central anti-nociceptive and pro-nociceptive pathways[32,214-216]. However, only few studies have investigated the effects of probiotics on visceral sensitivity in humans. Indeed, in healthy subjects a non-fermented milk product contained *Bifidobacterium animalis subsp Lactis*, *Streptococcus thermophiles*, *Lactobacillus bulgaricus* and *Lactococcus lactis subsp Lactis* was able to affect activity of brain regions that control central processing of emotion and sensation[81]; on the other hand, in IBS patients the multispecies probiotic Winclove 801 containing six bacterial species (*Bifidobacterium lactis* W52, *Lactobacillus casei* W56, *Lactobacillus salivarius* W57, *Lactococcus lactis* W58, *Lactobacillus acidophilus* NCFM and *Lactobacillus rhamnosus* W71) failed to ameliorate visceral hypersensitivity in comparison with placebo[217]. However, this limited information is insufficient to translate the animals findings to "hypersensitive" disease states involving disturbances in the gut/brain axis such as IBS.

Epithelial barrier/intestinal inflammation/immune activation

Despite IBS being considered as a "functional", non-organic disease, it is widely accepted that persistence of IBS-like symptoms occurs in a small percentage of patients after a documented episode of intestinal bacterial or viral infection. The fact that IBS could be a state of "low grade inflammation" is gaining acceptance based on the fact that animal and epidemiological studies indicate that IBS is characterized by an increased intestinal permeability, a biomarker of impaired epithelial barrier function. Further on, an increased activity of innate immune (mainly represented by accumulation of mast cells and antigen-presenting cells such as dendritic cells and macrophages) and an activated adaptive immune response in the intestinal mucosa and in blood, including an increased levels of systemic or mucosal cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-8, IL-12, associated to a decrease of anti-inflammatory cytokines (*i.e.*, IL-10) has been described in IBS[218-220]. Probiotics appear effective in reducing the inflammatory components of IBS. Thus, probiotics administered either as a single strain or in combination, maintain the integrity of intestinal epithelial barrier in inflammatory models[221-225]

and in humans[161,175], modulate both innate and adaptive immunity[226,227], restore the imbalanced ratio between pro-inflammatory and anti-inflammatory cytokines (*i.e.*, IL-10/IL-12)[154] and decrease the levels of pro-inflammatory cytokine as TNF-α and IFN-γ[228-230]. Human studies have demonstrated that a probiotic combination containing *Lactobacillus gasseri* KS-13, *Bifidobacterium bifidum* G9-1 and *Bifidobacterium longum* MM2 175 induced a less inflammatory cytokine profile in older adults[231], *Saccharomyces boulardii* supplementation induced a significant decrease in blood and tissue levels of proinflammatory cytokines IL-8 and TNF-α and an increase in anti-inflammatory IL-10 levels, as well as an increase in the tissue IL-10/IL-12 ratio ameliorating the quality of life of D-IBS[232] and the symptomatic response induced by *Bifidobacterium infantis* 35624 in IBS was associated with normalization of the ratio of an anti-inflammatory to a proinflammatory cytokine[154].

Luminal metabolism

The gut microbiota produces several metabolites including short-chain fatty acids (SCFAs), neurotransmitters, metabolites of bile acids, and cytokines that target enteric cells *via* specific receptors and signal to the brain *via* afferent vagal or endocrine pathways. SCFAs like acetate, propionate and butyrate derive from the fermentation of undigested and unabsorbed carbohydrates, *i.e.*, resistant starches and dietary fibers and are used as a fuel by the colonic microbiota. While propionate is largely taken up by the liver and acetate enters the systemic circulation to be metabolized by the peripheral tissues, butyrate works as major energy source for colonocytes. Butyrate modulates epithelial proliferation, apoptosis and cell differentiation in the large intestine[233], inhibits nuclear factor kappa B activation[234] and stimulates intestinal mucus production[235], thereby supporting the mucosal barrier function. Furthermore, butyrate plays a major role in inflammation-related repairs[236], offers protection against colonic carcinogenesis in rats[237] while in humans improves visceral perception[238], suggesting a possible beneficial effect in "hypersensitive" disorders such as IBS.

In contrast acetic and propionic acid-producing bacteria (*i.e.*, *Veionella* and *Lactobacilli* spp) have been reported in IBS patients[51] leading to enhanced production of SCFAs that are toxic at high concentrations and stimulate 5-HT release from the intestinal nerve endings[239]. As 5-HT initiates high-amplitude colonic contractions, accelerates intestinal transit and increases colonic motility, *i.e.*, all possible features of IBS, it might be speculated that these fermentation products play a role in IBS symptoms. However, fecal concentrations of SCFAs in IBS patients differ only slightly in comparison to healthy subjects[51,240] and IBS symptoms show only a slight correlation with SCFAs fecal concentrations. Moreover, studies that have examined the effect of probiotics supplementation on fecal SCFAs in humans have provided conflicting results. Thus, the probiotic *Lactobacillus paracasei* DG modulated fecal butyrate concentration in healthy subjects[241], while bifidobacteria-fermented milk increased fecal butyrate, propionate and short-chain fatty acid concentrations but ameliorated symptoms in patients affects by UC[242]. Finally, the *Bifidobacterium lactis* Bb12 increased the fecal level of acetate and lactate in preterm infants[243].

However studies demonstrating a strong correlation between the assumption of probiotics and the level of fecal SCFAs in IBS patients are lacking.

Dysfunction of the brain-gut axis and psychological distress

There are evidence that the gut microbiota modulates the CNS *via* the ENS, the ANS, the HPA axis and *vice versa*[124,213]: a deep correlation that influences both brain development and responses, intestinal motility, sensitivity, secretions and immunity. An intestinal dysbiosis may lead to alteration of brain-gut axis and probiotics may restore the normal interaction among all components of this pathway. Several beneficial actions of probiotics on brain-gut axis including the maintenance of intestinal barrier that "protects" the ENS, the effects of certain probiotics and their products on intestinal sensory and motor nerves of ENS, ANS and on several receptors (*i.e.*, opioid and cannabinoid receptors) and the modulation of cytokines profile leading to an anti-inflammatory action have been mentioned earlier in this review. Importantly, the HPA axis is a neuroendocrine system essential for the normal stress response to challenges in vertebrates which integrity is important in the pathogenesis of IBS[244,245]. There is evidence that certain probiotics directly influence the exaggerated HPA axis response observed in several experimental models of IBS. Indeed, *Bifidobacterium animalis subsp lactis* BB-12® and *Propionibacterium jensenii* 702 induced activation of neonatal stress pathways and an imbalance in gut microflora but also improved the immune environment of stressed animals and protected against stress-induced disturbances in adult gut microflora[246], while probiotic preparation containing live *Lactobacillus rhamnosus* strain R0011 and *Lactobacillus helveticus* strain R0052 improved gut dysfunction induced by maternal separation, at least in part by normalisation of HPA axis activity[247]. Moreover, probiotics alleviate anxiety- and depression-related behavior that are a typical feature of IBS, as *Lactobacillus rhamnosus* (JB-1) reduced the stress-induced elevation in corticosterone in stressed animals *via* modulation of GABA receptors implicated in anxiety behavior[216], *Bifidobacterium longum* 1714 had a positive impact on cognition in stressed mice[248] and the probiotic mixture VSL#3 induced an increase in brain-derived

neurotrophic factor (BDNF) expression and reduced age-related alterations in the hippocampus in a murine model of deterioration in cognitive functions[249].

Probiotics and gene expression: a new mechanism of action?

Finally, probiotics administration might result in a central (*i.e.*, CNS) and peripheral (*i.e.*, intestinal) reprogramming of genes. In the maternal separation (MS) rat model of IBS, *Bifidobacterium breve* 6330 influenced hippocampal BDNF gene expression[250], while the probiotic mixture VSL#3 downregulated the colonic expression of several genes (*i.e.*, TPH1, CCL2, CCR2, NOS3, NTRK1, BDKRB1, IL10, TNFRSF1B and TRPV4) encoding for proteins involved in nociception[214]; maternal probiotic intervention also increased the gene expression of ileal mucin-2 (MUC2)[230], indicating that the mechanism of action of probiotics is deeper and more complex than previously thought.

PREBIOTICS

The Food and Agriculture Organization of the United Nations (FAO) defines prebiotic as "a nonviable food component that confers a health benefit on the host associated with modulation of the microbiota" [251]. They represent an alternative strategy for reprogramming the gut microbiota by providing regular doses of a specific substrate engineered to be readily metabolized by specific desirable bacteria, thereby encouraging their growth in contrast to the development of harmful microbial species. Also known as "functional" foods[252], they escape absorption in the small bowel and enter the colon where they provide for nutrients for specific bacteria, mainly *bifidobacteria* and *lactobacilli*. Several prebiotics belong to the group of non-digestible carbohydrates: monosaccharides (*i.e.*, fructose), disaccharides (*i.e.*, lactose), oligosaccharides [(*i.e.*, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS)], and polyols (*e.g.*, sorbitol), the so-called Fermentable Oligo- Di- and Mono-saccharides And Polyols or FODMAPs, the prototype of which is inulin, a non-digestible carbohydrate naturally present in a large variety of plants that, when enzymatically hydrolyzed, produces oligofructose[252]. All of them occur in many fruits, cereals, vegetables and in human milk, which contains more than 1000 oligosaccharides[253]. Furthermore, long-chain polyunsaturated fatty acids have been tested as active prebiotics[254].

Experimental data[255,256] and human studies have shown a beneficial effect of prebiotic supplementation in different pathological conditions, including infections[254], allergies[257,258], pregnancy-related disorders[259-260], metabolic disorders[261,262], hepatic and gastrointestinal diseases including cirrhosis[263], IBD[264] and chronic constipation[265]. However, few studies have evaluated the efficacy of prebiotics in IBS and existing results are conflicting. Two trials, using oligofructose in one case[266] and fructo-oligosaccharides in the other[267], failed to confirm any beneficial effects of prebiotics, while two other studies have demonstrated symptoms improvement, with FOS ameliorating symptoms[268] and GOS lowering flatulence and bloating while also improving the anxiety score[83]. The clinical response may be dependent on the prebiotic type and dose, taking into account that low doses may be ineffective and high doses may stimulate colonic gas production. Indeed, some reports indicate that prebiotics may exacerbate IBS symptoms. Thus, lactulose and bran, that are successfully used for constipation, tend to produce substantial amounts of gas and abdominal pain and may aggravate symptoms of IBS[269-271]. Although fructose and sorbitol mixtures produce a modest increase in stool weight, they often increase flatulence and bloating in both healthy volunteers and IBS patients[272,273]. Ong *et al*[274] have recently shown that a diet rich in FODMAPs increases abdominal pain, bloating and flatulence in IBS patients, focusing the attention on the necessity of restricting fermentable carbohydrates in these patients[275-277]. Finally, fructose, sorbitol and a range of poorly absorbed polyhydric alcohols enter the gut where they retain a substantial amount of water causing unwanted diarrhea. However, low FODMAP diet recommended for reduction of IBS symptoms will have adverse effects on colonic luminal microenvironment and microbiota in healthy populations, reducing the absolute and relative bacterial abundance and diversity[278].

SYNBIOTICS

An important safety feature of probiotics is that they have a short lifespan within the gut and need repeated doses to keep a constant level. Indeed, an alternative strategy to maintain constant levels of beneficial bacteria within the gut is the contemporary administration of probiotic strains and prebiotics, the so-called synbiotic therapy. Few open label trials[64,279] and PCT studies have evaluated the efficacy of synbiotics in both functional pain[280] and IBS, all demonstrating a superiority of the probiotic/prebiotic combinations *vs* placebo[281-283]. Although the concept of combining prebiotic with probiotic is theoretically attractive, the limited experience and the poor quality of published studies do not allow any recommendation on their use in IBS.

ANTIBIOTICS

The alteration in the gut microbiota composition is increasingly considered to be a relevant pathogenetic factor in IBS, leading to the use of antibiotics as a treatment for this multifactorial syndrome. In addition, a SIBO may be relevant to at least a subset of IBS patients, thus justifying an antimicrobial approach.

However, it should be considered that lactose hydrogen breath test, routinely used as a surrogate marker for SIBO, has a low sensitivity and specificity[284-286]. The first antibiotic investigated in a clinical trial was neomycin, which is not adsorbed in the gut. Pimentel *et al*[285] have demonstrated that neomycin caused a 50% improvement in global IBS symptoms compared to placebo-treated patients, but also induced a rapid clinical resistance. Other broad-spectrum antibiotics reducing bacterial overgrowth have been challenged in the treatment of IBS, including tetracycline, amoxicillin clavulanate, metronidazole and fluoroquinolones such as norfloxacin[287,288]; however, these drugs are absorbable causing local and systemic side effects and their use in IBS patients is not recommended. The semisynthetic, antibacterial, rifamycin-derivative rifaximin has virtually no systemic absorption, is effective in improving IBS symptoms with low bacterial resistance profile and has a favorable side-effects profile.Since the first trial published almost a decade ago[289], several trials have shown that rifaximin is an effective treatment for IBS. The strongest evidence comes from two large-scale, multicenter studies: TARGET 1 and TARGET 2[290], which included a total of 1260 patient affected by IBS without constipation. In these phase 3, double-blind, placebo-controlled trials, significantly more patients in the rifaximin group than in the placebo group had adequate relief of global IBS symptoms during the first 4 wk of treatment (40.7% *vs* 31.7% respectively in the two studies combined). In addiction, more patients in the rifaximin group than in the placebo group had adequate relief of bloating, abdominal pain and stool consistency (40.2% *vs* 30.2% respectively in the two studies combined). The incidence of adverse events was similar. In general, available metanalysis tend to show a beneficial profile of rifaximin *vs* placebo on global IBS symptoms and bloating[291]. Furthermore, a pooled analysis by Schoenfeld *et al*[292] has confirmed the safety and tolerability profile of rifaximin in comparison with placebo, with similar incidence of drug-related adverse events. In conclusion, rifaximin is the only antibiotic which can play a role in the treatment of IBS, but limitations should be considered: (1) it is effective in less than 50% of patients; (2) large phase Ⅲ study has included only patients without constipation, indeed studies on C-IBS are needed; (3) its long-term effects have not been investigated; and (4) the effect of rifaximin on gut microbiota composition is essentially unknown.

FECAL TRANSPLANTATION IN IBS: A NEW LIFE FOR AN OLD TREATMENT?

Fecal microbiota transplant (FMT) is an interesting strategy proposed for a large spectrum of diseases[293], including recurrent *Clostridium Difficile* infection (CDI) resistant to conventional antibiotic therapies (that represents the main gastroenterological indication for FMT), chronic constipation, IBD, recurrent metabolic syndrome, multiple sclerosis, autism and chronic fatigue syndrome. A systematic review of 325 cases of FMT for CDI suggested a lower success rate for upper gut administration (76%), as compared with colonoscopy (89%) and enema (95%) administration[294]. Moreover, a large case series of CDI patients has showed rapid response and a cure rate of nearly 90%, without significant adverse event rate[295]. Colonic infusion of donor human intestinal flora can reverse UC in a small series of selected patients, determining endoscopic and histological remission[296]. This report was followed by a number of small studies of FMT in children and adults with UC, CD, and pouchitis with mixed results[297-302]. It has been recently demonstrated that sensitivity to colonic distension of IBS patients can be transferred to rats by the fecal microbiota transplant[303].

From a pure technical point of view, FMT is an easy technique that requires a healthy donor (usually a patient's family member or an anonymous donor), with no risk factors for transmissible diseases or any issues that may alter the cellular composition, particularly prior antibiotic use. The FMT Working Group have recently published guidelines for FMT donor selection criteria and screening tests[304]. The steps for an adequate treatment of the fecal material include dilution in saline solutions, homogenization and filtration, while the route of administration can be naso-duodenal, transcolonoscopic or enema based. Naso-duodenal administration is the method patients favour the lest. Colonoscopy allows direct assessment of the colonic mucosa for the assessment of disease severity and exclusion of coexistent pathology. Enema administration is effective, cheap and safe and carries less procedural or institutional admission costs.

To date, only anecdotal data have been reported about the efficacy of FMT for IBS treatment, results being far conclusive. The first case series on FMT in IBS has been published in 1989 by Borody *et al*[305] demonstrating approximately a 50% of relief in symptoms of IBS after FMT; however, the study also included IBD and CDI patients and did not show any distinction within the results between these different diseases. Since that publication, the lead Author has treated with FMT more than 300 D-IBS patients whose symptoms had failed to respond to conventional therapies[306]. Other preliminary studies indicate a beneficial effect in patients with chronic constipation (*i.e.*, relief in defecation and reduction of bloating)[307], but these data need confirmation in rigorous clinical trials. Pinn *et al*[308] have reported promising results using FMT in IBS patients with refractory disease: 70% of the patients achieving resolution or improvement of the symptoms. Taken together, these data indicate that FMT is a promising treatment option for serious and recurrent CDI[309,310]. However, many questions

should be answered before it may be recommended as routine standard treatment of IBS[311] and randomized, double-blinded, placebo-controlled trials are required.

CONCLUSION

Evidence regarding the manipulation of gut microbiota composition as an effective cure for IBS is increasing and, to date, probiotic supplementation and antimicrobial therapy with not absorbable antibiotics are promising treatment. However, all meta-analysis point out to the weakness of the majority of the studies and recommend additional RCT trials to confirm the positive findings reported by small studies. Specifically, additional information on type of probiotic, doses, side effects and time of administration are required, as well as data on patient subtypes. Prebiotics are often burdened by unwanted side effects and synbiotics lack of sufficient numbers of clinical trials on their efficacy and safeness. FMT might be a reasonable option for treating IBS, as it is an inexpensive and easy treatment, but standardized controlled trials are necessary to ascertain which patients are eligible, the most effective regiment as well as the most acceptable method of administration of the donor's microbiota. For these therapeutic options, a careful selection of patients, a close monitoring of clinical data and side effects and a long-term follow-up are necessary, as well as more information on modification of host microbiota composition.

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TOPIC HIGHLIGHT

2016 Irritable Bowel Syndrome: Global view

Inflammation in irritable bowel syndrome: Myth or new treatment target?

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Abstract

Low-grade intestinal inflammation plays a key role in the pathophysiology of irritable bowel syndrome (IBS), and this role is likely to be multifactorial. The aim of this review was to summarize the evidence on the spectrum of mucosal inflammation in IBS, highlighting the relationship of this inflammation to the pathophysiology of IBS and its connection to clinical practice. We carried out a bibliographic search in Medline and the Cochrane Library for the period of January 1966 to December 2014, focusing on publications describing an interaction between inflammation and IBS. Several evidences demonstrate microscopic and molecular abnormalities

in IBS patients. Understanding the mechanisms underlying low-grade inflammation in IBS may help to design clinical trials to test the efficacy and safety of drugs that target this pathophysiologic mechanism.

Key words: Inflammation; Irritable bowel syndrome; Mast cells; Neuroendocrine cells; Pathology

Core tip: Low-grade intestinal inflammation plays a key role in the pathophysiology of irritable bowel syndrome, and this influence is likely multifactorial. Several evidences showed microscopic and molecular abnormalities in large subsets of patients with irritable bowel syndrome. Understanding the mechanisms underlying the low-grade inflammation in this disease may help to design clinical trials to test the efficacy and safety of drugs that target this pathophysiologic mechanism.

Sinagra E, Pompei G, Tomasello G, Cappello F, Morreale GC, Amvrosiadis G, Rossi F, Lo Monte AI, Rizzo AG, Raimondo D. Inflammation in irritable bowel syndrome: Myth or new treatment target? *World J Gastroenterol* 2016; 22(7): 2242-2255 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2242.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2242

INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic, relapsing, and remitting functional disorder of the gastrointestinal tract characterized by abdominal pain, bloating, and changes in bowel habits that lack a known structural or anatomic explanation[1].

IBS consists of a set of altered bowel habits over a period of time and includes abdominal pain and discomfort. IBS is one of the most common diagnoses in primary care, accounting for approximately 12% of all visits[2]. In addition, a survey conducted by Russo *et al*[3] found IBS to be the most common functional gastrointestinal diagnosis, comprising 35% of all outpatient referrals to gastroenterologists[2,3]. Therefore, IBS is also the most common diagnosis for gastroenterologists, accounting for 20%-50% of patient visits[2,4].

With regard to the sex-related prevalence of IBS, in Western countries, the prevalence of IBS in female patients outnumbers that in male patients by 2:1[5,6]. Furthermore, the ratio of female to male IBS sufferers in the non-patient population is 2:1, and within the patient population who seek consultations with primary care physicians, females outnumber male patients by 3:1[5,7]. Finally, in tertiary-care settings, the number of female IBS patients is four- to five-times higher than the number of males[5-8]. This prevalence should not only be strictly attributed to sex, but also to gender-related differences in healthcare-seeking behavior and sociocultural characteristics that vary between men and women with IBS as well as among different cultures[5,6].

According to the Rome Ⅲ criteria, IBS is defined based on the presence of recurrent abdominal pain or discomfort at least three days per month in the past three months associated with two or more of the following: (1) improvement with defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool. These criteria should be fulfilled for the previous three months with symptom onset at least six months before diagnosis[9].

Rome Ⅲ criteria subtype IBS according to the predominant bowel habit as IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed type, and unclassified[9]. To this end, the definition of bowel-habit type is based on the patient's description of the stool form by referring to the Bristol Stool Scale[10]. Furthermore, IBS patients can be divided into two categories: sporadic (nonspecific) and postinfectious (PI-IBS) inflammatory bowel disease-associated[11,12].

IBS symptoms cannot be explained by structural abnormalities, and specific laboratory tests or biomarkers are not available for IBS. Therefore, IBS is classified as a functional disorder whose diagnosis depends on the history of manifested symptoms[13].

The cause of IBS is unknown, but a single factor is not likely to be responsible for the several presentations of this complex disorder[1]; new fields of research in this area include mucosal inflammation, postinfectious low-grade inflammation, genetic and immunologic factors, alteration of the human microbiota, alterations of the intestinal permeability, and dietary and neuroendocrine factors[13]. Usually, routine histologic examinations do not show significant colonic mucosal abnormalities in the majority of IBS patients; however, recent quantitative histologic, immunohistochemical, and ultrastructural analyses have indicated subtle organic alterations in these patients.

This literature review aims to summarize the findings relating the spectrum of mucosal inflammation to IBS, highlighting their relationship to the pathophysiology of IBS and their connections, if any, to clinical practice.

RESEARCH

We carried out a bibliographic search in MEDLINE for the period of January 1966 to July 2015, and focused on identifying publications describing an interaction between inflammation and IBS. The keywords used were: irritable bowel syndrome, inflammation, mucosal inflammation, pathology, mast cells, neuroendocrine cells, immune cells, intestinal permeability, and enteric nerves. The inclusion criteria to select articles were based on design (systematic reviews, meta-analysis,

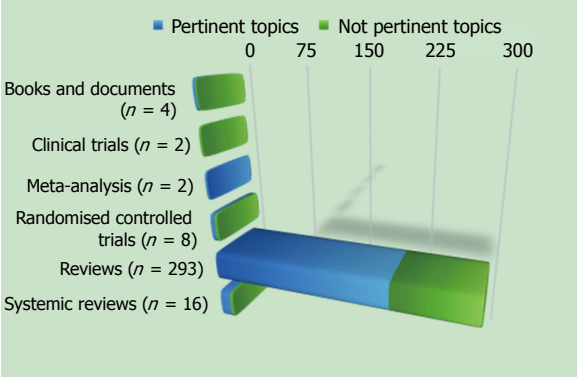

Figure 1 Literature findings on the relationship between irritable bowel syndrome and inflammation (*n* = 305).

clinical trials, and experimental studies on animals) and population (adult patients > 18 years of age). We excluded articles not pertinent to this topic.

According to the aforementioned criteria, we found 305 studies, and we excluded 100 studies because they were not pertinent to this topic (Figure 1).

ROLE OF ENDOSCOPY IN IBS

According to the American Gastroenterology Association, "a colonoscopy is recommended for patients over age 50 years (due to higher pretest probability of colon cancer), but in younger patients, performing a colonoscopy or sigmoidoscopy is determined by clinical features suggestive of disease (*e.g.*, diarrhea, weight loss), and may not be indicated"[14].

However, the British guidelines suggest that, "given the high frequency of colonic cancer in the population at large, an examination of the colon is advisable for a change in bowel habit over the age of 50"; the authors of these guidelines highlighted that, "as IBS patients have no increased risk of colon cancer, advice on screening for this is no different from the general population"[15].

More recently, Japanese guidelines suggested that "colonoscopy has a diagnostic value, not only for excluding organic diseases but also for supporting the existence of pathophysiology compatible to IBS due to visceral hypersensitivity to colonoscopic procedures and colonic spasms"[16].

A prospective, multicenter study performed by Ishihara and coworkers[17] aimed to determine the presence of organic colonic lesions in IBS patients. Their study showed that the prevalence of organic colonic diseases in IBS patients was at an acceptably low level, thus showing that the Rome Ⅲ criteria are specific for the diagnosis of IBS. Conversely, another study performed by Hsiao and coworkers[18] demonstrated that IBS was not associated with the development of colon cancer in Taiwan.

Despite these recommendations, a recent Korean survey indicated that colonoscopy was the most commonly required test (79.5%) in IBS patients[19], whereas a study performed by Lieberman and coworkers[20] to evaluate trends in the utilization and outcomes of colonoscopy in the United States from 2000 to 2011 showed that the most common reason for colonoscopy in patients aged < 50 years was the evaluation of symptoms, such as IBS (28.7%), together with bleeding or anemia (35.3%).

Based on these updated data, IBS still represents the majority of colonoscopic biopsies seen by pathologists[21] that are usually considered either normal or near to normal on routine histologic examination. These findings provide valuable information to the physician who is suspecting a diagnosis of IBS. However, the pathologists must be aware of variations in normal tissue as well as artifacts that may result from bowel preparations or the biopsy procedure in order to not to report these variations as abnormal. Furthermore, the pathologists must consider subtle morphologic changes reported in the intestinal mucosa in IBS and associated with chronic inflammatory cells, mast cells, enteroendocrine cells, and enteric nerves[22].

INFLAMMATION IN IBS

The intestinal mucosa harbors a florid immune system that can be regarded as "physiologically inflamed"[23]. Thus low-grade inflammation can only be evaluated using quantitative assessments[24]. IBS patients have been shown to exhibit significant increases in lamina propria immune cells in the colonic mucosa compared with healthy subjects, which appears to be more predominant in the right than in the left colon[25].

Granulocytes and plasma cells

More than ten years ago, O'Sullivan *et al*[26] evaluated the number of plasma cells, lymphocytes, eosinophils, neutrophils, and macrophages in a case-control study. Specifically, each cell type was semiquantitatively graded in hematoxylin-and-eosin-stained sections of the entire colon, and possible increases in the number of mast cells (MCs) in the colon of IBS patients compared with controls were examined using a monoclonal mouse antibody for human MC tryptase (AA1). Other than MCs, increases in cellular infiltrate were not observed in the IBS group, and the number of MCs was significantly increased in the cecum of IBS patients compared with controls.

Similarly, in 2008, Piche and coworkers[27] aimed to examine associations between fatigue, depression, and the MCs of the colonic mucosa in IBS by comparing the numbers of CD3-positive intraepithelial T lymphocytes, MCs, plasma cells, eosinophils, and neutrophils in cecal biopsies taken during colonoscopy. There was not a significant difference in the numbers of intraepithelial lymphocytes, plasma cells, eosinophils, or neutrophils between IBS patients and healthy controls, but the MC numbers per high-power field were significantly higher

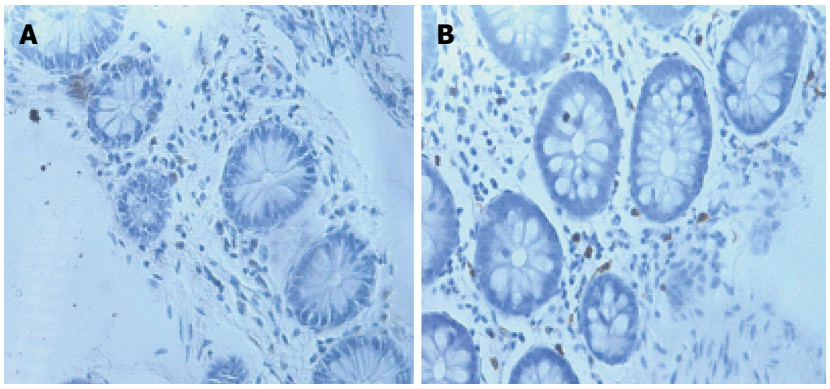

Figure 2 Immunohistochemistry for tryptase showing increases in the number of mast cells in the colonic mucosa in inflammatory bowel disease. A: Irritable bowel syndrome patient; B: Control (× 40 magnification). Courtesy of Giancarlo Pompei, personal data.

in IBS patients than in healthy controls (9.3 *vs* 4.0, $P = 0.001$). Furthermore, the number of MCs correlated with the severity of fatigue and depression scores in IBS patients but not in healthy controls[28].

With regard to the small bowel, Walker *et al*[29] examined the MC, eosinophil, and intraepithelial lymphocyte populations in duodenal biopsies of subjects with IBS and functional dyspepsia. Their study showed a significant increase in the number of intraepithelial lymphocytes in biopsies from the duodenum in patients with IBS-C. However, this increase was not observed in the second part of the duodenum. Nevertheless, MC counts were also higher in IBS cases in both the first and second parts of the duodenum, but this difference was only significant for constipation-predominant IBS[28]. Interestingly, the eosinophil counts in this study did not differ between IBS patients and controls in either the first or second part of the duodenum[28].

To date, a significant difference in the numbers of plasma cells, neutrophils, or eosinophils has not been demonstrated among IBS cases[28]. Importantly, increases in eosinophils were not identified in IBS, but the eosinophil counts were elevated in individuals with functional dyspepsia[29]. Functional dyspepsia and IBS demonstrate significant overlap in cross-sectional surveys[30], despite attempts to classify them separately, and a biomarker to predict the presence of IBS remains elusive[31]. Therefore, this histopathologic marker may serve to distinguish the two conditions[28].

MCs

MCs are innate immune cells involved in food allergies, wound healing, and protection against pathogens[32]. Their functional activation consists of a degranulation process, leading to the release of various compounds, such as histamine, tryptase, and chymase[32].

More than 40 years ago, Hiatt and Katz[33] were the first to demonstrate MC infiltration in the muscular layer of full-thickness colonic biopsy samples from four patients with "spastic colitis"[34]. Increases in the number of mucosal MCs have been observed in IBS patients in the rectum[35,36], rectosigmoid colon[37,38], descending colon[39-44], ascending colon[36], cecum[45], terminal ileum[25,36,46], jejunum[47-49], and duodenum[29,50] (Figure 2).

However, discrepancies in data obtained from these studies could be due to sex-specific differences, bowel preparation artifacts, fixation protocols, tissue orientation, sample size, or IBS-related recruitment criteria[32,43,44,51]. Furthermore, clinical studies have also yielded conflicting evidence correlating MC numbers with the onset of abdominal pain[32].

The number of functionally active MCs (exhibiting changes in the release of tryptase and histamine), rather than the absolute number of MCs, plays a pivotal role in IBS[43,50]. Consequently, the role of MCs in IBS may be affected by cells that are functionally active and form close connections with enteric and extrinsic nerve terminals, thus determining visceral hypersensitivity and altered gut function[47].

Lymphocytes

The aforementioned inflammatory changes described in the mucosa of IBS patients show that immune activation may play a role in IBS pathophysiology[34]. Mucosal T- and B-type lymphocytes are also part of the gut adaptive immune response to pathogens[32].

An increased density of T lymphocytes in the mucosa of IBS patients has been widely demonstrated. Specifically, the T-cell density is higher in the rectum[35,36,52], rectosigmoid colon[53], colon[38,48,54], cecum[26], jejunum[55], and duodenum[56]. Like for MCs, several studies showed that IBS patients exhibit a normal lymphocyte density in different intestinal tissue segments[19,36,38,53,57-61]. These discrepancies may be due to differences in the immunostaining techniques, quantification methods, and IBS-related recruitment criteria (Figure 3)[32].

Conversely, the density of B-cells in the rectum[19,36], colon[19,43,53,62], cecum[19,20], ileum[36], or jejunum[60] did not differ in IBS patients. However, Forshammar and coworkers[62] and others[32] found a decrease in secretory B cells in the colon (Figure 4).

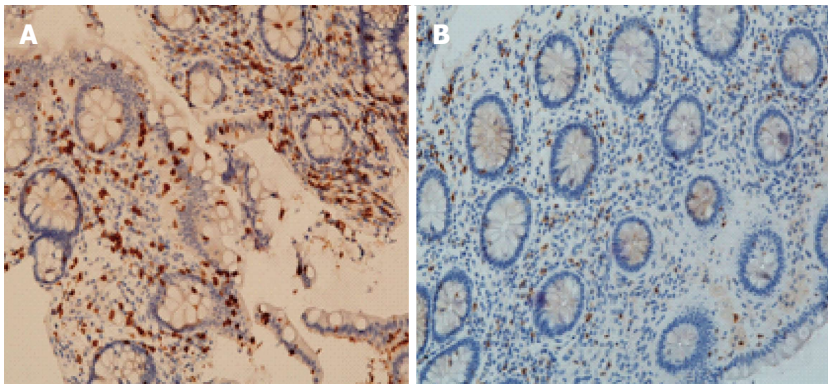

Figure 3 Immunohistochemistry for CD3 showing increase in the number of intraepithelial T-lymphocytes in the large bowel in inflammatory bowel disease. A: Irritable bowel syndrome patient; B: Normal distribution of T-lymphocytes, which are mainly distributed within the lamina propria of the large bowel of a control patient (× 20 magnification). Courtesy of Giancarlo Pompei, personal data.

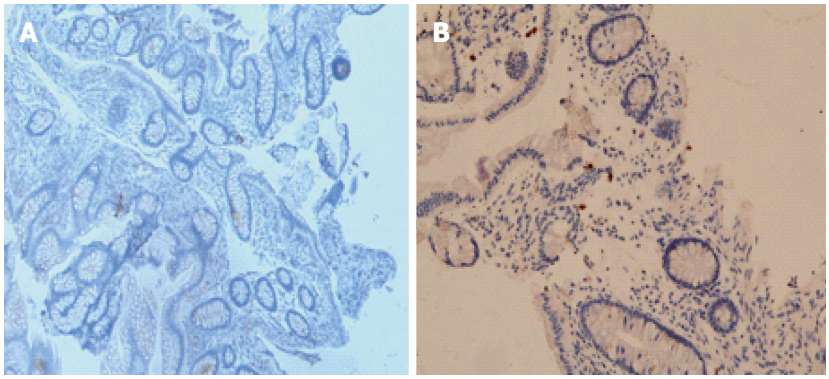

Figure 4 Immunohistochemical staining for CD20. An equivalent distribution of B lymphocytes in the lamina propria was seen in the large intestine. A: Patient with irritable bowel syndrome; B: Control patient (× 20 magnification). Courtesy of Giancarlo Pompei, personal data.

With regard to intraepithelial lymphocytes, several discrepancies have been reported in studies assessing the density of these cells in IBS patients. An increase in density has been demonstrated in the rectum[35,36,57], colon[57], jejunum[55,49], and duodenum[56], but these increases were not confirmed by other groups[20,32,45,49,59,61].

Dendritic cells and macrophages

Dendritic cells are antigen-presenting cells that are usually located at the surveillance interfaces of the human body, such as the skin or mucosa, and play a pivotal role in the generation and regulation of immune responses[63]. In fact, they represent the link between allergen uptake and the clinical manifestations of intestinal inflammation[64,65]. Furthermore, the gut also harbors abundant macrophages. These cells do not function as typical antigen-presenting cells and lack the cellular machinery for the production of pro-inflammatory cytokines and induction of potent adaptive immune responses. However, they show very potent phagocytic activity[65].

In a *Trichinella spiralis* mouse model of PI-IBS, Long and coworkers[66] reported numerical and phenotypic alterations in the lamina propria dendritic cells following acute *T. spiralis* infection. In their study, the lamina propria dendritic cells expressed increased levels of costimulatory molecules and exhibited a greater ability to migrate and induce $CD4^+$ T-cell proliferation[66]. Consequently, these changes favored increased levels of pro-inflammatory interferon-γ, interleukin-23, and tumor necrosis factor-α production in the so-called "PI-IBS stage"[66,67].

With regard to macrophages, the numbers of resident $CD68^+$ macrophages are reduced in PI-IBS cases following *Campylobacter jejuni* infection, probably due to the cytotoxic nature of the pathogen inside host cells[50]. Similarly, *Shigella* spp.[68,69] and *Salmonella* infections have also been shown to be involved in PI-IBS, and both of these organisms are intracellular pathogens that induce phagocytosis by macrophages[70,71]. Furthermore, *Salmonella* seems to be less cytotoxic to macrophages[72] and also causes a marked interleukin-18 response[72] with important implications in exerting paracrine effects on surrounding immune cells (inducing interferon-γ expression). These changes result in increased levels of activated T cells in the infected intestine[47,52,54,55,57].

Enteroendocrine cells

Enteroendocrine cells (residing among the epithelial

cells of the mucosa in all gut segments, with the exception of the esophagus) secrete multiple regulatory molecules that control several functions, such as postprandial secretion and motility[12,73,74]. Animal experimental studies have demonstrated abnormalities in the function of enteroendocrine cells in the setting of gastrointestinal infection[73]. Enteroendocrine cells seem to be involved in visceral hypersensitivity, disturbed gastrointestinal motility, and abnormal gut secretion[12] that patients with IBS usually present[75-77].

In fact, visceral hypersensitivity has been shown in the colon of IBS patients[78-85], but the correlation of this disturbance with the severity of abdominal pain is currently poorly understood[12]. Some authors hypothesize the involvement of a peripheral mechanism in visceral hypersensitivity in IBS[86-88]. Because the gut mucosa can produce high levels of serotonin[88], a reduction in serotonin impairs intracellular uptake and degradation in the gut epithelial cells and consequently increases serotonin availability of in the gut mucosa[89-92]. Therefore, the amount of serotonin available at its receptors is markedly increased[12,87,88]. Due to this mechanism, the development of visceral hypersensitivity in PI-IBS patients may be due to the increase in serotonin at the 5-hydroxytryptamine 3 receptors of the sensory neurons of the enteric nervous system.

Dysmotility has also been shown in the small and large bowel of IBS patients, as evidenced by the involvement of cholecystokinin, ghrelin, secretin, serotonin, and peptide YY[12]. Both esophageal motility abnormalities and abnormal gastric emptying have been observed in IBS patients with conflicting results[93-108]. Specifically, IBS-C patients exhibit delayed gastric emptying, whereas accelerated gastric emptying was observed in IBS-D patients[77,98]. In this setting, ghrelin was shown to stimulate gastric and small- and large-bowel motility[106-117]. Conversely, serotonin relaxes the stomach through a nitrergic pathway and consequently delays gastric emptying[118-120]. Moreover, cholecystokinin[121-123] and secretin[124,125] were shown to relax the proximal stomach, which inhibited gastric emptying in a manner similar to secretin; furthermore, small-bowel transit was also found to be delayed overall in IBS-C patients and accelerated in IBS-D patients[126-131], but conflicting results have been reported[132-139].

Ghrelin, which is involved in the stimulation of small-bowel motility, and peptide YY, a regulator of the ileal brakes[140-145], play pivotal roles in gastric emptying by stimulating the absorption of water and electrolytes and inhibiting prostaglandin E2 and vasoactive intestinal polypeptide[146-148]. Therefore, ghrelin cell density is reportedly low in the stomach, and that of peptide YY is reported to be high in the ileal mucosa of IBS-C patients, whereas the ghrelin cell density is reported to be high in the stomach, and that of secretin is reported to be low in the duodenum of IBS-D patients[12]. Furthermore, colorectal transit was found to be delayed in IBS-C patients and accelerated in IBS-D patients[80,125,126,149-154], but contradictory results have been reported[12,105,152,154-176].

Finally, abnormal gastrointestinal secretion is common in IBS patients. Among the abnormalities in the enteroendocrine cells in IBS patients, low levels of duodenal cholecystokinin (which stimulates the secretion of digestive enzymes from pancreatic exocrine glands) and secretin (which stimulates pancreatic bicarbonate and fluid secretions)[122,123], as well as high levels of ileal peptide YY (which stimulates the absorption of water and electrolytes), were reported in IBS-C patients[12].

Intestinal permeability

The term "mucosal barrier" was adopted by Cummings *et al*[177] in 2004 to describe "the complex structure that separates the internal milieu from the luminal environment, consisting of the vascular endothelium, the epithelial cell lining, and the mucus layer, next to which digestive secretions, immune molecules, cell products such as cytokines, inflammatory mediators, and antimicrobial peptides, are found, mainly produced by Paneth cells in the crypts of the small intestine"[177,178].

Conversely, impaired intestinal permeability is defined as "an altered permeability being nontransiently changed compared to the normal permeability leading to a loss of intestinal homeostasis, functional impairments and disease"[178]. Increases in the numbers of MCs in the gut of IBS patients were found to be related to changes in gut permeability[32,179-181]. In IBS patients, the assessment of permeability via the urinary recovery of orally administered markers has demonstrated increases in the permeability of the small and in the large bowels[45,182-184], but results have been contradictory[185-187]. Furthermore, rectal permeability was also reportedly increased in IBS-D patients following exposure to MC tryptase[188]. Finally, recent studies of the permeability of the epithelial barrier have reported a decrease in the colonic expression of the tight junction proteins occludin, claudin-1, and zonula occludens-1 in IBS patients[42,189] (Figure 5).

Several aliments, as well as microbiota and bile acids, have been proposed to cause low-grade inflammation and altered permeability in IBS. In fact, in some patients, IBS was related to food allergy[190]. Furthermore, endogenous triggers, such as MC-derived histamine, proteases, and eicosanoids, could increase intestinal permeability, either directly or via the stimulation of neurons of the enteric nervous system[183]. Moreover, serotonin was also identified as an endogenous trigger of pain, inflammation, and increased permeability in IBS[40]; therefore, LX1031, an oral inhibitor of tryptophan hydroxylase, the principal enzyme needed for mucosal serotonin synthesis, has been successful for treatment of patients with non-

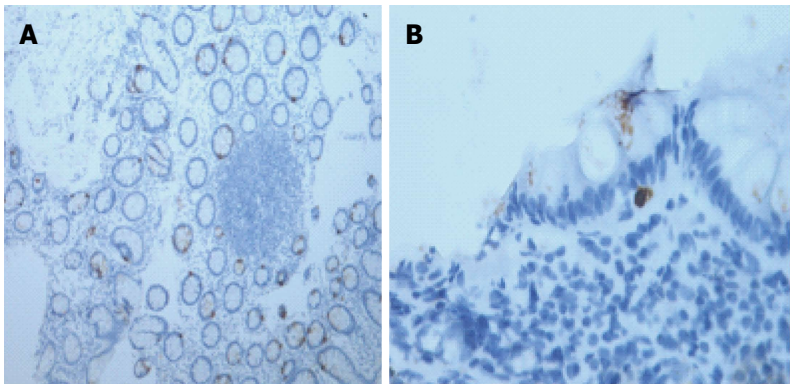

Figure 5 Immunohistochemistry for chromogranin A showing increased expression in nerve terminals at the level of the basal membrane in the large intestine in inflammatory bowel disease. A: Irritable bowel syndrome patient; B: Control patient (× 40 magnification). Courtesy of Giancarlo Pompei, personal data.

constipating IBS[191].

Enteric nerves

Few studies have investigated the role of calcitonin gene related peptide and substance P. Wang *et al*[37] investigated the incidence of IBS in patients who had recovered from bacillary dysentery by focusing on neuroimmunologic changes, including changes in interleukins, MCs, neuropeptides, and the relationship between MCs and intestinal nerves[50]. The density of substance P-immunoreactive fibers was increased in both the ileal and the rectosigmoid samples of IBS patients[37], but the density of calcitonin gene related peptide-containing fibers remained unchanged. Palsson and coworkers[192] reported similar findings, and Kerkhoffs and coworkers[193] reported an increase of rectal substance P. However, these findings have not been universal[192,194], possibly reflecting region-specific discrepancies[51].

Neuronal plasticity in the enteric nervous system has also been investigated[195]. Akbar *et al*[38] investigated the capsaicin receptor transient receptor potential vanilloid 1-immunoreactive nerve fibers in colonic biopsies from patients with IBS. Specifically, they demonstrated that the number of nerve fibers exhibiting immunoreactivity for substance P and transient receptor potential vanilloid 1 was increased in IBS patients. Moreover, the number of these fibers did not differ by IBS subtype, but significantly correlated with patient pain scores[50,196].

Nerve growth factor has also been suggested to play a central role in promoting the growth and differentiation of primary afferent fibers[50]. Specifically, its expression was found to be markedly increased in rectal biopsies from pediatric[197] and adult IBS patients[198], suggesting both the sprouting of sensory afferent fibers expressing transient receptor potential vanilloid 1 and increases in receptor sensitivity in IBS, which consequently induced visceral hyperalgesia[50]. More recently, Dothel *et al*[199] also showed that nerve fiber density and sprouting, as well as the expression of nerve growth and neurotrophic tyrosine kinase receptor type 1, are significantly increased in the mucosal tissues of patients with IBS. Mucosal mediators participate in these neuroplastic changes.

Finally, the morphology of enteric glia, which are known to regulate intestinal barrier integrity and neuronal activity[200] has only been examined in one study of human intestinal biopsy samples from IBS patients and was found to be unchanged[50,201].

CONCLUSION

Low-grade intestinal inflammation plays a key role in the pathophysiology of IBS, and this role is likely multifactorial[202]. Several studies demonstrated microscopic and molecular abnormalities in IBS patients[202,203].

The above-reported evidence provides a rationale to test the efficacy of intestinal anti-inflammatory compounds in patients with IBS. Previously, treatment with corticosteroids was found to be ineffective in PI-IBS patients[204]; however, MC stabilizers have produced promising results, particularly in IBS-D, suggesting that immune mechanisms and MCs are involved in the generation of IBS symptoms[205,206]. Based on this approach, Clarke and coworkers[207] recently conducted a phase 3, multicenter, tertiary setting, randomized, double-blind, placebo-controlled trial in patients with Rome Ⅲ-confirmed IBS to evaluate the efficacy and safety of mesalazine in patients with IBS. In this study, mesalazine treatment was not superior to placebo based on the study primary endpoint (68.6% *vs* 67.4%, 95%CI: 12.8-15.1, P = 0.870). However, the placebo response was high in this trial and this study enrolled both male and female subjects and patients with mild symptoms[207], which likely masked drug efficacy. Furthermore, a subgroup of patients with IBS showed a sustained therapy response and benefits from mesalazine therapy[207].

As mentioned above, abnormalities in the enteric nervous system of the gut may alter digestion, gastrointestinal motility, and visceral hypersensitivity, which contribute to symptom onset and play a pivotal

role in the pathogenesis of IBS[12].

The enteric nervous system of the gut seems to be affected by genetic differences, diet, intestinal flora, and inflammation[12]. For example, the food content of FODMAPs and fibers, which interacts with the intestinal flora and drives subsequent fermentation, may increase intestinal osmotic pressure to induce hormonal and serotonin release[12]. Targeting these known factors may improve the control of IBS symptoms by acting on mechanisms that trigger these symptoms and regulate the pathophysiology of IBS. Finally, probiotics have also been found to be effective in select IBS patients, as suggested by several recent systematic reviews, guidelines and meta-analyses, by improving intestinal permeability[208-212].

In conclusion, a high proportion of IBS patients show low-grade inflammation, which is a multifactorial process, in the intestinal mucosa. Understanding the mechanisms underlying the low-grade inflammation in IBS may allow the design of clinical trials that test the efficacy and safety of drugs that target the pathophysiologic mechanism of this disease.

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REVIEW

Management of pancreatic fluid collections: A comprehensive review of the literature

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Abstract

Pancreatic fluid collections (PFCs) are a frequent complication of pancreatitis. It is important to classify PFCs to guide management. The revised Atlanta criteria classifies PFCs as acute or chronic, with chronic fluid collections subdivided into pseudocysts and walled-off pancreatic necrosis (WOPN). Establishing adequate nutritional support is an essential step in the management of PFCs. Early attempts at oral feeding can be trialed in patients with mild pancreatitis. Enteral feeding should be implemented in patients with moderate to severe pancreatitis. Jejunal feeding remains the preferred route of enteral nutrition. Symptomatic PFCs require drainage; options include surgical, percutaneous, or endoscopic approaches. With the advent of newer and more advanced endoscopic tools and expertise, and an associated reduction in health care costs, minimally invasive endoscopic drainage has become the preferable approach. An endoscopic ultrasonography-guided approach using a seldinger technique is the preferred endoscopic approach. Both plastic stents and metal stents are efficacious and safe; however, metal stents may offer an advantage, especially in infected pseudocysts and in WOPN. Direct endoscopic necrosectomy is often required in WOPN. Lumen apposing metal stents that allow for direct endoscopic necrosectomy and debridement through the stent lumen are preferred in these patients. Endoscopic retrograde cholangio pancreatography with pancreatic duct (PD) exploration should be performed concurrent to PFC drainage. PD disruption is associated with an increased severity of pancreatitis, an increased risk of recurrent attacks of pancreatitis and long-term complications, and a decreased rate of PFC resolution after drainage. Any pancreatic ductal disruption should be bridged with endoscopic stenting.

Key words: Pancreatic fluid collection; Pancreatic fluid

collection; Pseudocyst; Walled-off pancreatic necrosis; Walled-off pancreatic necrosis; Pancreatitis

Core tip: Pancreatic fluid collections are a frequent complication of pancreatitis. Management includes correctly classifying these collections, initiating early enteral feeding, and draining symptomatic collections. Endoscopic ultrasound with stent placement is the technique of choice. Both metal and plastic stents are efficacious, though metal stents may offer an advantage. When necrosis is present within the collection, direct endoscopic necrosectomy may be required in addition to drainage. Lumen apposing metal stents allow for direct endoscopic necrosectomy through the stent and are preferred in these patients. When a pancreatic duct leak is suspected, endoscopic investigation and stenting is mandated.

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INTRODUCTION

Pancreatic fluid collections (PFCs) area frequent complication of pancreatitis. It is estimated that 5%-15% of pancreatitis episodes are complicated by the development of pseudocysts[1]. PFCs can also result from inflammation due to trauma, post surgery, post transplant or pancreatic ductal obstruction. Fifteen percent of pancreatitis episodes are complicated by pancreatic necrosis, and approximately 33% (range 16%-47%) of those with necrosis are complicated by infected necrosis[2]. Management of these collections can pose a challenge. Traditionally, the management has primarily been surgical. However, with new understanding of the pathophysiology paired with new technological advancements, the pendulum has swung towards an emphasis on a minimally invasive approach with a progression to more invasive options as necessary.

CLASSIFICATION OF PANCREATIC FLUID COLLECTIONS

Correctly classifying PFCs is critical for optimizing treatment and management. The first widespread classification system was developed in 1993 by an international consensus meeting in Atlanta, Georgia and became referred to as the Atlanta Criteria[3]. This criteria classified pancreatic fluid collections as acute or chronic collections, with chronic collections being further divided into pancreatic necrosis, pseudocysts, and pancreatic abscesses.

However, with improving pathophysiologic understanding and improving diagnostic tools, it became clear that a more detailed organizational system was required. More specifically, one that distinguished between collections containing fluid alone *vs* those arising from necrosis and/or containing solid components. As such, a new classification system was developed known as the revised Atlanta criteria[4]. Similar to the original Atlanta Criteria, PFCs are classified as acute (< 4 wk after the pancreatitis episode) or chronic (> 4 wk after the pancreatitis episode). However, in the revised criteria, both acute and chronic collections are further subdivided based on the presence of necrosis within the collection. Acute collections are divided into: acute peripancreatic fluid collections (APFC) and acute necrotic collections (ANC); chronic fluid collections are divided into: pseudocysts or walled-off pancreatic necrosis (WOPN). These new classifications are important because the treatment and management varies depending on the type of collection.

ENTERAL FEEDING

The first step in the management of any PFC is ensuring adequate nutritional support. In mild to moderate acute pancreatitis, oral feeding can be initiated when symptoms are controlled. In severe cases, patients have traditionally been kept nil per os (npo) due to concerns for worsening pancreatic inflammation if normal pancreatic digestion were to be enacted during oral intake[5]. However, prolonged npo in the catabolic stress state of pancreatitis leads to a negative nitrogen balance and nutritional deficiency that became recognized to be associated with a higher mortality rate due to loss of function and structural integrity of vital organs[6]. As a result, total parental nutrition (TPN) became the standard of care in patients with severe acute pancreatitis in an attempt to avoid pancreatic stimulation while still providing nutritional support[5,6].

ENTERAL FEEDING *VS* TPN

This approach was questioned when studies began showing that complete bowel rest is associated with intestinal mucosal atrophy leading to increased intestinal permeability and bacterial translocation[7]. Furthermore, a metabolically deprived gut absorbs endotoxins and other bacterial products stimulating endogenous cytokines which increases the likelihood of nosocomial infections, sepsis, and organ failure[8]. The use of TPN was further called into question with the emergence of data showing that enteral feeding distal to the ligament of Treitz causes negligible pancreatic stimulation and therefore may be safe in patients with severe pancreatitis[9].

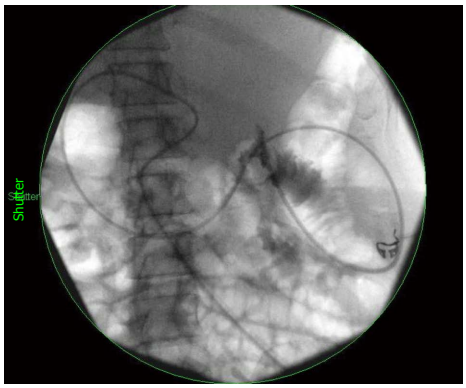

Figure 1 Fluoroscopic image of a percutaneous nasojejunal feeding tube beyond the ligament of Treitz.

In 2010, the Cochrane Collaboration published their results of a meta-analysis comparing randomized trials of enteral nutrition *vs* TPN in patients with severe acute pancreatitis[6]. Eternal nutrition was associated with a significant reduction in mortality, multi-system organ failure, and systemic infections with a trend towards shorter length of hospital stay. Based on these findings, enteral nutrition was recommended as the standard of care for nutritional support in acute pancreatitis[6].

In addition to improved morbidity and mortality rates, enteral nutrition is associated with a lower overall cost compared to TPN. In a study of 24 patients with severe acute pancreatitis, enteral nutrition was associated with savings of $5553.06 per patient ($P$ = 0.08). Though not statistically significant, there was a medium to large effect size (d = 0.61) suggesting that the difference between the two groups would likely have been significantin a larger sample size[10].

EARLY *VS* LATE ENTERAL FEEDING

The timing of initiation of enteral feeding in severe acute pancreatitis has been debated. In a recent meta-analysis, patients receiving early initiation of enteral nutrition (defined as within 48 h of admission) had significantly lower rates of infectious complications (OR = 0.45, 95%CI: 0.15-0.77, P < 0.05), organ failure (OR = 0.27, 95%CI: 0.14-0.50, P < 0.05), length of hospitalization [mean difference -2.18 d, 95%CI: (-3.48)-(-0.87), P < 0.05], and mortality (OR = 0.31, 95%CI: 0.14-0.71, P < 0.05) compared to those with delayed enteral nutrition or TPN[11]. However, the exact time at whichenteral feeding should be initiated is not yet established.

JEJUNAL *VS* GASTRIC ENTERAL FEEDING

Though enteral nutrition distal to the ligament of Treitz is thought to decrease pancreatic stimulation, placement of a nasojejunal tube requires endoscopy for placement and is more cumbersome than a nasogastric tube which can be placed bedside (Figure 1). Studies have been performed to evaluate the safety of nasogastric feeds compared to nasojejunal feeds. A meta-analysis of these studies showed no difference in mortality and tolerance between the two types of feeding; however, this analysis was limited by the small sample size (157 patients in the 3 studies included for analysis) and the lack of verification of placement of the nasojejunal tube distal to ligament of Treitz in two of the three studies[12]. A recent non-inferiority trial of 78 patients randomized to nasogastric or radiologically-confirmed nasojejunal feeding was recently published, showing non-inferiority of nasogastric feeds. However, there was a higher rate of infectious complications, need for surgical intervention for infected necrosis, and mortality in the nasogastric feeding group[13]. A prospective, randomized controlled trial evaluating nasogastric *vs* nasojejunal feeding called the Study on Nutrition in Acute Pancreatitis is currently underway, which will provide further evidence on this subject. Until more high-quality data is available, nasojejunal feeding remains the preferred route of enteral nutrition.

ENTERAL FEEDING FORMULATIONS

Enteral nutrition is available in a variety of formulations, including standard, elemental, and semi-elemental with the latter two more commonly used based upon the assumption that they result in less pancreatic stimulation. Standard enteral formulas are, however, significantly cheaper and proven effective[14]. Windsor *et al*[15] randomized patients with severe acute pancreatitis to TPN *vs* standard enteral formulas and found that patients receiving standard enteral formulas had improved clinical outcomes compared to those receiving TPN, including decreased rates of systemic inflammatory response syndrome, sepsis, and multi-system organ failure. Makola *et al*[14] also examined the efficacy of enteral formulas in acute pancreatitis and demonstrated that it is associated with an improvement in the severity of pancreatitis, a higher albumin, and a trend towards a normal BMI.

INDICATIONS FOR DRAINAGE OF PFCS

In the initial Atlanta criteria, PFCs were recommended for drainage based on the presence of symptoms and/or complications such as abdominal pain, gastrointestinal obstruction, vascular compression, biliary obstruction, or infection, as well as on the size of the collection. However, in the revised criteria, size alone does not necessitate treatment; only symptomatic PFCs are recommended for drainage. Historically, drainage has been managed *via* surgical techniques. But with the advent of newer and more advanced endoscopic tools and expertise, and an associated reduction in health care costs, minimally invasive endoscopic drainage has

become the preferable approach[16].

PANCREATIC PSEUDOCYSTS

As described in the revised Atlanta criteria, a pseudocyst is an encapsulated fluid collection, without the presence of solid debris, that develops as a consequence of pancreatitis a minimum of 4 wk after the initial injury[3].

SURGICAL DRAINAGE

Surgical cystogastrostomy involves an open or laparoscopic procedure in which an anastomosis is created between the lumen of the cyst cavity and the stomach or small bowel using suturing or stapling devices[11]. Depending on the location, a cystojejunostomy can also be a surgical alternative.

Historically, surgical drainage was an efficacious therapy, with published pseudocyst recurrence rates between 2.5%-5% post-drainage, but complication rates approaching 30% in some studies[16]. As endoscopic therapies emerged, initial studies comparing surgical cystogastrostomy to endoscopic cystogastrostomy showed grossly equivalent success rates, defined as pseudocyst resolution, and comparable complication rates[17,18]. However, as endoscopic techniques improved, endoscopic therapy became the preferred initial treatment approach. A randomized comparative trial by Varadarajulu *et al*[19] looking at surgical *vs* endoscopic cystogastrostomy found that while the two techniques yielded similar technical success and complication rates, endoscopic therapy was associated with a shorter hospital stay, a lower overall cost, and better mental health and physical health component scores among patients.

PERCUTANEOUS DRAINAGE

Percutaneous drainage involves placement of an external drainage catheter into the pseudocyst using real-time imaging guidance, usually with computed tomography (CT) or ultrasound (US) with fluoroscopy. Initial studies comparing surgical drainage to percutaneous drainage found both procedures to be efficacious[20,21]. However, more recent comparative studies have generally favored percutaneous drainage[22], with some studies even demonstrating a mortality benefit[23]. Percutaneous drainage has also recently been compared to endoscopic drainage. A recent study directly comparing percutaneous *vs* endoscopic management retrospectively reviewed 81 patients. This study found equal technical success rates and adverse events rates between the techniques, but a decreased re-intervention rate, a shorter hospital stay, and a decreased number of follow-up abdominal imaging studies among patients drained endoscopically[24].

CONVENTIONAL TRANSMURAL DRAINAGE

Conventional transmural drainage was the endoscopic procedure of choice to drain PFCs in the early era of endoscopic PFC management. This procedure consists of endoscopically visualizing the PFC bulge in the gastric wall, creating a fistulous tract between the pseudocyst cavity and the gastric lumen using a seldinger technique, advancing a guidewire into the pseudocyst cavity, dilating the tract, and finally deploying one or more plastic stents to secure apposition and allow for continuous drainage[25].

This concept was first introduced into the medical literature in 1975 in a case report by Rogers *et al*[26]. It was expanded upon by Kozarek *et al*[27] in 1985 in a case series of 4 patients who underwent endoscopic cystogastrostomy needle decompression and by Cremer *et al*[28] in 1986 in which they described 13 patients who underwent cystogastrostomy with transnasal drain placement. The first large series evaluating this technique was published in 1989 and consisted of a 7-year follow-up study of 33 patients who underwent endoscopic cystogastrostomy or cystoduodenostomy with a success rate of 82%, recurrence rate of 12%, and complication rate of 2%[29]. In 1995, Binmoeller *et al*[30] published a similar study of 53 patients with a success rate of 87%, recurrent rate of 21%, and complication rate of 11%. A series of subsequent studies from the early 2000s demonstrated similar results, reporting success rates between 70%-100% and complication rates ranging from 2%-40%, mainly bleeding, perforation, and infection due to stent occlusion or migration[29-39].

One of the limitations of this technique was the need for the PFC to be bulging into the gastric wall. It is estimated that no bulge is present in 42%-48% of PFCs, limiting the efficacy and safety of this technique in almost half of all cases[40]. However, with the incorporation of echoendoscopy, this limitation was overcome.

EUS-GUIDED TRANSMURAL DRAINAGE

The use of EUS in pseudocyst drainage provides endoscopists with the ability to identify and avoid vascular structures between the cyst and the gastric lumen, to measure the distance between the lumen and the cystic lesion and ensure that adequate apposition can be obtained, to localize non-bulging pseudocysts that are otherwise unidentifiable using endoscopy alone, and to confirm the lack of solid or necrotic components within the pseudocyst cavity (Figure 2). This technique first emerged in the medical literature in 1992 by Grimm *et al*[41] and 1996 by

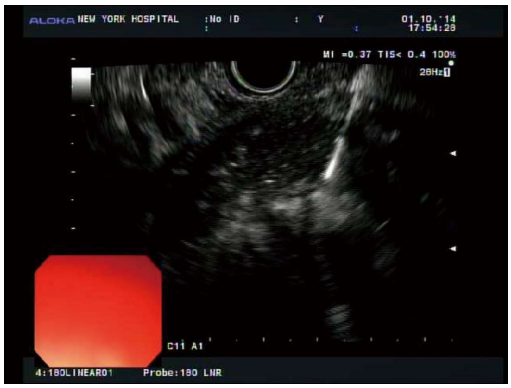

Figure 2 Endosonographic visualization of accessing a pancreatic fluid collection with a fine needle aspiration needle.

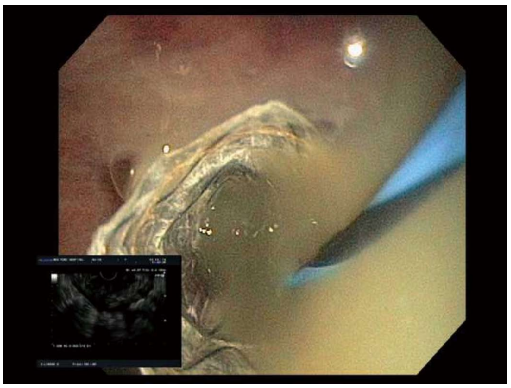

Figure 3 Endoscopic visualization of a biliary fully covered self-expanding metal stent to drain an infected pseudocyst.

Wiersema[42], both of whom described a single case of successful endoscopic pseudocyst drainage using an echoendoscope. Several larger case series looking at 27 patients[30] and 35 patients[43] documented success rates of 78% and 89% with complication rates of 7% and 4% respectively, significantly lower than with conventional transmural drainage (CTD). Since then, a multitude of studies have validated these initial findings, with early studies quoting success rates ranging from 80%-100% and complication rates averaging around 10%, mainly bleeding and perforation[25,30,35,40,43-47].

More recent studies have further subdivided pancreatic pseudocysts into simple *vs* infected pseudocysts. Sadik *et al*[48] noted a 94% success rate and 5% complication rate in simple pseudocysts *vs* 80% success rate and 30% complication rate in infected pseudocysts. Similarly, Varadarajulu *et al*[49] found a 93.5% success rate and 5% complication rate *vs* a 63% success rate and 16% complication rate in sterile *vs* infected pseudocysts. This suggests that while EUS-guided drainage is still efficacious, infected pseudocysts are more difficult to drain and associated with a higher complication rate.

Several studies have directly compared EUS-guided PFC drainage to CTD. A study by Kahaleh *et al*[25] showed equal efficacy and safety between the two techniques when conventional drainage was used for bulging lesions and EUS-guided drainage was used for all other lesions. Subsequently, two prospective, randomized studies by Varadarajulu *et al*[50] and Park *et al*[51] found significantly higher technical success rates with EUS-guided drainage, and a trend towards a better safety profile although statistical significance was not reached.

FULLY COVERED SELF-EXPANDING METAL STENTS

Fully-covered self-expanding metal stents (FCSEMS) offer a variety of advantages over traditional plastic stents. Firstly, they allow for a larger drainage lumen, which decreases the risk of stent occlusion and theoretically the need for repeat procedures. And secondly, they allow for shorter procedure times since they require a single access of the cyst for deployment, rather than the multiple access points required for the deployment of multiple plastic stents.

A study by Penn *et al*[52] looked at 20 patients with symptomatic pancreatic pseudocysts which were drained under EUS guidance with placement of biliary FCSEMS (Wallflex; Boston Scientific, Natick, MA). They found a 100% technical success rate and a 70% rate of complete pseudocyst resolution without recurrence. Three patients experienced complications (15%) requiring surgery in 2 of the 3, and stent migration was noted in 3 patients, all of whom still achieved pseudocyst resolution. Similarly, a case series looking at 18 patients with symptomatic pseudocysts drained with FCSEMS (Wallflex; Boston Scientific) under EUS-guidance showed a 78% rate of complete pseudocyst resolution (14 patients); however, 16% of patients required surgery for ongoing sepsis or ineffective drainage[53]. A case series looking at 20 patients with infected pseudocysts drained with biliary FCSEMS and/or esophageal CSEMS reported a 100% technical success rate and a complete clinical success rate of 85%[54]. In this series, 1 patient had stent migration and 1 patient had a superinfection treated with medical therapy[55].

FCSEMS with antimigratory fins (Viabil, Conmed, city, state) have also been proven efficacious (Figure 3). Talreja *et al*[56] reported a 78% clinical success rate with complete resolution after pseudocyst drainage in 18 patients. In their series, 1 patient had stent migration, though still achieved pseudocyst resolution. Berzosa *et al*[57] evaluated the same stent for pseudocyst drainage in 5 patients and found an 83% resolution rate without recurrence at 18 wk.

PLASTIC STENTS *VS* METAL STENTS

Despite the advantages that FCSEMS hold over traditional plastic stents, direct comparison has not

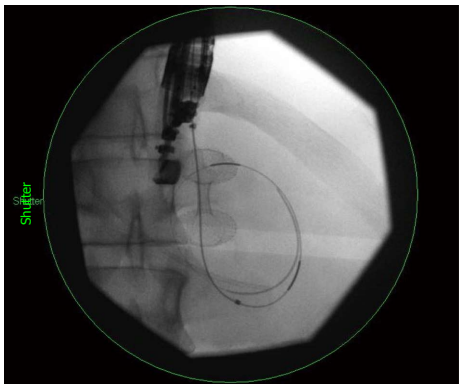

Figure 4 Fluoroscopic visualization of a lumen apposing metal stent being deployed into a pancreatic pseudocyst.

consistently shown them to be superior. A recent meta-analysis that included 698 patients found no difference in treatment success, adverse events, or recurrence rates between pseudocysts drained with multiple plastic stents *vs* metal stents[18,58]. However, a more recent study by Sharaiha *et al*[59] of 230 patients found that pseudocysts drained with plastic stents were 2.5 times more likely to report adverse events than when FCSEMS were used. Similarly, complete pseudocyst resolution was 89% with plastic stents compared to 98% with FCSEMS. Given the cost differential between metal and plastic stents, further randomized controlled trials are needed prior to final recommendations on the best approach.

NOVEL LUMEN-APPOSING METAL STENT

In 2013, a new FCSEMS received FDA approval for use in drainage of PFCs (Axios; Boston Scientific, Boston, MA). The design of the stent includes two 21 mm or 24 mm flanges on either side of a 10 mm or 15 mm diameter lumen to help decrease the risk of stent migration. The first clinical data using this stent came from a study by Itoi *et al*[60] in 2012 looking at 15 patients with symptomatic pseudocysts. Success rate in the trial was 100%, with zero percent recurrence at 11 mo follow-up and the only complication being stent migration in 1 patient without clinical sequelae (Figure 4).

Several additional studies validated this initial reported success. A prospective study by Shah *et al*[61] looking at 33 patients found a technical success rate of 91% with a cyst resolution rate of 93%. Gornals *et al*[62] looked at 9 patients who underwent pseudocyst drainage with placement of a lumen-apposing metal stent (LAMS) and reported a technical success 89%, a pseudocyst resolution rate of 100%, and 1 significant complication (pneumothorax). Walter *et al*[63] published their data of 15 patients with a clinical success rate of 93%, resolution rate of 100%, and 1 significant complication (perforation). And most recently, Rinninella *et al*[64] documented a 98% cyst resolution rate with adverse events in 2 out of 41 patients.

In summary, pancreatic pseudocysts can be efficaciously managed endoscopically. Although conventional endoscopic drainage can be safely used for bulging pseudocysts, the majority of pseudocysts are drained under EUS-guidance to allow for safer access and a decrease in complications. Metal stents, including the newly emerged lumen-apposing metal stent, carry an advantage over plastic stents in pseudocyst drainage, but given the increased cost and lack of definitive evidence as to their superiority, further trials are needed (Table 1).

MANAGEMENT OF WOPN

WOPN is a PFC that contains solid necrotic debris surrounded by a clearly defined capsule with or without concurrent fluid[4]. Although a small percentage of WOPN will resolve spontaneously, the majority of collections will require drainage.

SURGICAL DRAINAGE

Open surgical debridement has historically been the therapy for WOPN[65,66]. Surgical management consists of 4 principal approaches, all involving accessing the pancreatic bed but differing in the surgical approach. The standard approaches include access *via* the lesser sac, the gastrocolic-omentum, or trans-mesenterially through the transverse mesocolon[67]. Once the necrosectomy has been performed, the options are: (1) necrosectomy alongside open packing[68]; (2) planed, staged re-laparotomies with repeat lavage[69]; (3) closed continuous lavage of the lesser sac and retro-peritoneum[65]; and (4) closed packing[70].

Open necrosectomy is associated with high morbidity (34% to 95%) and mortality (6% to 25%) rates[71-76], and a plethora of adverse events including organ failure, perforation, wound infections, hemorrhage, chronic pancreatico-cutaneous and entero-cutaneous fistulae, and abdominal wall hernias[65,67,70,72,73]. With the development of laparoscopic surgery, minimally invasive procedures supplanted open debridement as the surgical option of choice. Laparoscopic debridement can be performed using 2 approaches: trans-peritoneal (anterior) or retroperitoneal (posterior)[66]. The trans-peritoneal approach involves an anterior access through the stomach or the bowel to drain the collection. The retroperitoneal approach uses a mini-lumbotomy, usually left-sided, through which a laparoscope is introduced to remove the necrotic debris under direct visualization. Currently, the trans-peritoneal approach is rarely used due to increased technical difficulty and the risk of contamination of the peritoneal cavity[77]. Additionally, a retroperitoneal approach can be performed with minimal or no gas insufflation and avoids the complications associated with severing the peritoneum[78,79].

Table 1 Pancreatic fluid collection

	Cases	Procedure used	Device used	Clinical success rates	Technical success rates	Complications
Pancreatic pseudocysts						
Hookey *et al*[35], 2006	116	Conventional Transmural drainage	Stents	88%	88%	11% complication rate
Antillon *et al*[40], 2006	33	EUS-Guided Transmural drainage	Double-pigtail Stent	94%	82%	2 major complications and 3 minor complications
Azar *et al*[44], 2006	23	EUS-Guided Transmural drainage	Double-pigtail Stent	91%	91%	
Lopes *et al*[46], 2007	51	EUS-guided Transmural drainage	Straight/Double-pigtail Stent	94%	94%	17.7% stent migration, stent obstruction
Barthet *et al*[45], 2008	50	EUS-guided Transmural drainage	Double-pigtail Stent/ Straight Polyethylene	90%	98%	18% morbidity, 5 superinfections
Talreja *et al*[56], 2008	18	EUS-guided drainage	Covered self-expanding metal stent	95%	78%	Superinfection (5), bleeding (2), and inner migration (1).
Berzosa *et al*[57], 2012	7	Single-step endoscopic ultrasonography-guided drainage	Single-self expandable metal stent	100%	83%	
Fabbri *et al*[95], 2012	22	EUS-guided drainage	Covered self-expanding metal stent	77%	77%	
Penn *et al*[52], 2012	20	EUS-guided drainage	Fully covered self-expandable metallic stents	70%	70%	Pseudocyst infection (2), post transmural drainage fever and post-ERCP pancreatitis(1)
Itoi *et al*[60], 2012	15	EUS-guided drainage	Novel lumen-apposing, self-expandable metal stent (Axios)	100%	100%	
Weilert *et al*[53], 2012	18	EUS-guided drainage	Fully covered self-expanding metal stent	78%	78%	
Varadarajulu *et al*[19], 2013	20	Endoscopic Cystogastrostomy	Plastic stents	95%	90%	
Sarkaria *et al*[97], 2014	17	EUS-guided drainage	Fully covered esophageal self-expandable metallic stents	88%	88%	Perforation during tract dilation (1)
Shah *et al*[61], 2015	33	EUS-guided drainage	Lumen-apposing, covered, self-expanding metal stent; Axios	91%	93%	Abdominal pain (n = 3), spontaneous stent migration, back pain (n = 1), access-site infection, and stent dislodgement (n = 1)
Walter *et al*[63], 2015	61	EUS-guided drainage	Axios	93%	98%	stent migration (n = 3), stent dislodgement during necrosectomy (n = 3), stent removal during surgery (n = 2), or refusal by the patient (n = 2)
Mukai *et al*[99], 2015	2	EUS-guided drainage/ Direct endoscopic necrosectomy	novel flared-type biflangedmetal stent	100%	100%	There was 1 psuedocyst recurrence in cystogramy
Rinninella *et al*[64], 2015	18	EUS-guided drainage	Lumen-apposing, self-expanding metal stent (Axios)	100%		
Sharaiha *et al*[59], 2015	230	EUS-guided transmural drainage	118 DP plastic stents/112 FCSEMS	75%-90%	< 90%	
Walled-off Necrosis						
Seewald *et al*[89], 2005	13	Direct endoscopic necrosectomy	Double-pigtail stent	91%	91%	Minor bleeding after balloon dilation, Necrosectomy (4)
Charnley *et al*[92], 2006	13	Direct endoscopic necrosectomy	Double-pigtail stents	92.3%	92.3%	
Voermans *et al*[90], 2007	25	Direct endoscopic necrosectomy	Double-pigtail stents	93%	93%	Surgery(2), Hemorrhage (1), perforation of cyst wall (1)
Papachristou *et al*[88], 2007	53	Direct endoscopic necrosectomy	Double-pigtail stents	81%	81%	Twelve patients (23%) required open operative intervention a median of 47 d (range, 5–540) after initial endoscopic drainage/debridement, due to persistence of WOPN (n = 3), recurrence of a fluid collection (n = 2), cutaneous fistula formation (n = 2), or technical failure, persistence of pancreatic pain, colonic obstruction, perforation, and flank abscess (n = 1 each)

Escourrou *et al*[91], 2008	13	Direct endoscopic necrosectomy	Double-pigtail stents	100%	100%	bleeding (n = 3), transient aggravation of sepsis (n = 3)
Seifert *et al*[93], 2009	93	Transmural endoscopic necresectomy	Multiple Stents	80%	80%	Bleeding (13), Perforations of the necrosis (5), fistula formation (2), air embolism (2), complications at other organs (2)
Gardner *et al*[102], 2009	45	25 used direct endoscopic neserectomy and 20 used conventional standard endoscopic drainage	Multiple Stents	45%	88% for DEN and 45% for Standard endoscopy drainage	
Gardner *et al*[94], 2011	104	Direct endoscopic necrosectomy	Multiple Stents	91%	91%	14%; included 5 retrogastric perforations/pneumoperitoneum
Attam *et al*[98], 2014	10	endoscopic transluminal necrosectomy and transmural drain	Novel large-bore, fully covered metal through-the-scope esophageal stent	90%	100%	
Smoczyński *et al*[100], 2014	112	Endoscopic drainage	Multiple Stents	84%	93%	Stoma bleeding (19), GI Perforation (4), collection perforation (2), sepsis (1), stent migration (3)
Sarkaria *et al*[97], 2014	17	EUS-guided drainage	Fully covered esophageal self-expandable metallic stents	83%	83%	
Mukai *et al*[99], 2015	19	EUS-guided drainage and DEN for PFCs using the novel flared-type BFMS	novel flared-type biflangedmetal stent	100%	100%	
Rinninella *et al*[64], 2015	52	EUS guidance FCSEMS/ LACSEMS	Axios Stent	90.4%	100%	3 patients required surgery due to infection/1 patient had a perforated wall
Walter *et al*[63], 2015	46	EUS guided drainage	Axios Stent	81%	81%	9%

EUS: Endoscopic ultrasonography; GI: Gastrointestinal; DEN: Direct endoscopic necrosectomy; PFCs: Pancreatic fluid collections.

PERCUTANOUS DRAINAGE

Percutaneous drainage for WOPN involves placement of a catheter into the collection under US guidance with fluoroscopy or CT guidance. Ideally, a retroperitoneal approach is taken. After placement and aspiration of as much fluid as possible, 12 French drains are left in place and irrigated with 10-20 mL of sterile saline 3 times daily. The catheters can be upsized to a maximum of 28 French as the patient's follow-up requires[80].

Traditionally, the success rate of percutaneous drainage alone (defined as survival without the need for additional surgical necrosectomy) ranged from 35%-84%, with mortality rates ranging from 5.6%-34% and morbidity ranges of 11%-42%, most commonly due to pancreatico-cutaneous fistulas and pancreatico-enteric fistulas which occur in an as many as 20% of cases[81-85]. Consequently, percutaneous drainage is more often used as an adjunct therapy, often serving as the first step of a step-up approach to endoscopic or surgical drainage[65,76,81]. The Dutch PANTER trial illustrated this concept by comparing open necrosectomy with a less-invasive step-up approach in 88 patients[86]. In the step-up approach, patients first underwent percutaneous drainage of the collection followed by minimally invasive retroperitoneal necrosectomy if clinical improvement was not achieved. Results showed that the minimally invasive approach was associated with an overall decreased mortality rate, fewer major and long-term complications, and reduced overall healthcare costs. Of note, percutaneous drainage alone without subsequent necrosectomy was achieved only in 30% of patients.

ENDOSCOPIC NECROSECTOMY

The endoscopic technique for drainage of WOPN is called direct endoscopic necrosectomy (DEN). As in pseudocyst drainage, EUS is used to identify and access the collection, a wire is coiled within the cavity lumen, and the fistulous tract is created. However, unlike pseudocyst drainage, the tract is then dilated enough to allow for passage of an endoscope into the collection. Mechanical cleaning with removal of necrotic debris is then performed.

Nasocystic drainage is typically performed to facilitate liquefaction of the debris and improve drainage[31].

Hydrogen peroxide (H_2O_2) can be used to facilitate removal of necrotic debris[15]. H_2O_2 is infused into the cavity during endoscopy in a 1:5 or 1:10 dilution with normal saline, allowing for enhanced necrotic tissue dislodgement and debris extraction during endoscopy. The use of H_2O_2 has been shown to decrease procedure time, reduce complication rates, and decrease the total number of necrosectomy sessions until resolution. Some adverse events have been reported including bleeding, perforation, and self-limited pneumoperitoneum. However, these complications are rare, especially after the incorporation of carbon dioxide for peri-procedural insufflation.

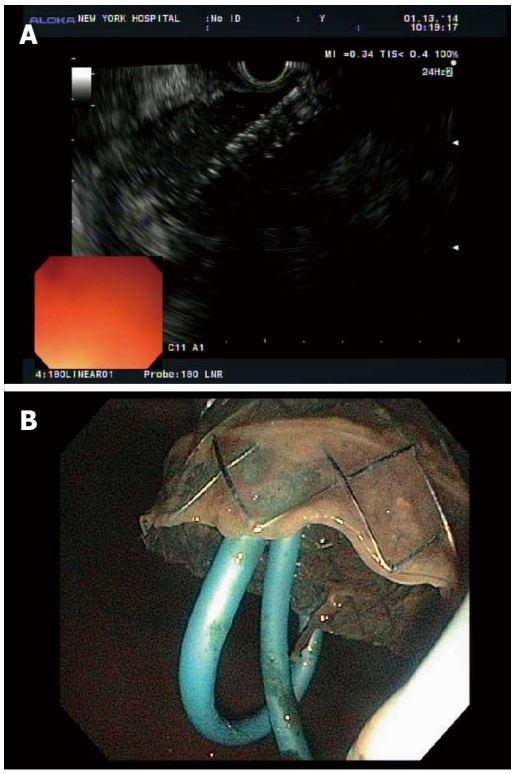

Figure 5 Endosonographic visualization (A) and endoscopic visualization (B) of a biliary fully covered self-expanding metal stent being deployed into walled-off pancreatic necrosis.

The first experiences with endoscopic necrosectomy were done through the deployment of plastic stents and placement of a nasocystic drain without direct mechanical debridement. This was first described by Baron *et al*[87] in 1996, in which 11 patients underwent WOPN drainage with an overall success rate of 81% and a complication rate of 36% (bleeding and infection). Papachristou *et al*[88] reported similar findings in 2007 in a study of 53 patients, with an overall success rate of 81% and a complication rate of 21%.

Seewald *et al*[89] introduced the concept of dilation of the fistulous tract to allow for advancement of an endoscope into the necrotic cavity and mechanical removal of debris. They described a 91% WOPN resolution rate in 13 patients, with 2 patients having recurrence on 4 mo follow-up necessitating surgical resection. Voermans *et al*[90] documented a 93% success rate in 25 patients, with only 2 patients requiring surgical intervention for bleeding and perforation. Smaller studies by Escourrou *et al*[91] and Charnley *et al*[92] found similar results.

The first multicenter study evaluating endoscopic necrosectomy was performed by Seifert *et al*[93]. In this study of 93 patients, an 80% clinical success rate was achieved with a 23% complication rate and 7.5% mortality rate. A second multicenter study was published by Gardner *et al*[94] in 2011 looking at 104 patients with WOPN. Successful resolution was achieved in 91% of patients, with a complication rate of 14% including 3 patients requiring surgical intervention either for bleeding or failed resolution, 5 patients dying of other causes prior to WOPN resolution, and 1 peri-procedural death due to hypotension.

FULLY-COVERED SELF-EXPANDING METAL STENTS

Biliary FCSEMS provide a larger stent lumen for drainage of WOPN, but are limited in that they do not permit passage of an endoscope (Figure 5). Fabbri *et al*[95] published results of 2 patients with WOPN drained with biliary FCSEMS (Wallflex, Boston Scientific). In 1 patient, the WOPN completely resolved; in the second patient, the stent migrated leading to widespread sepsis and need for surgical intervention. Berzosa *et al*[57] also looked at 2 patients with WOPN drained with biliary FCSEMS (Viable, ConMed). The WOPN resolved in both patients with no recurrence after 18 wk follow-up.

Esophageal FCSEMS have a larger lumen diameter and allow for passage of the endoscope through the lumen of the stent after deployment (Figure 6A). The first reported case of WOPN drainage using an esophageal FCSEMS was published by Antillon *et al*[96]. Sarkaria *et al*[97] published results of 17 patients who underwent WOPN drainage with placement of an esophageal stent, 88% of whom demonstrated complete resolution with an average of 5 endoscopic sessions and 2 of whom ultimately required surgical intervention. No major complications were reported. Attam *et al*[98] found similar results in 10 patients using a through-the-scope esophageal FCSEMS in which resolution was achieved in 90% of patients after an average of 3 endoscopic sessions. Two patients required stent revision due to persistent infection in long-term follow-up, and 1 patient died of gastrointestinal bleeding from a pseudoaneurysm. Esophageal FCSEMS are a promising concept in the endoscopic management of WOPN. However, the development of lumen apposing metal stent may supplant the utilization of esophageal FCSEMS.

LAMS

The previously mentioned LAMS (Axios, Xlumena) also allows for passage of an endoscope through the lumen of the stent into the cavity for mechanical necrosectomy. Only a small number of studies have been published specifically evaluating the use of LAMS for drainage of WOPN. Shah *et al*[61] achieved WOPN resolution in 10 of 11 patients using a LAMS for drainage. Walter *et al*[63] looked at 46 patients with WOPN and found a clinical success rate of 81%, with an overall major complication rate of 9% due to infection from stent occlusion, all managed endoscopically with only 3 patients ultimately requiring surgical intervention for persistent infection. Most recently, Rinninella *et al*[64] documented a 90% clinical

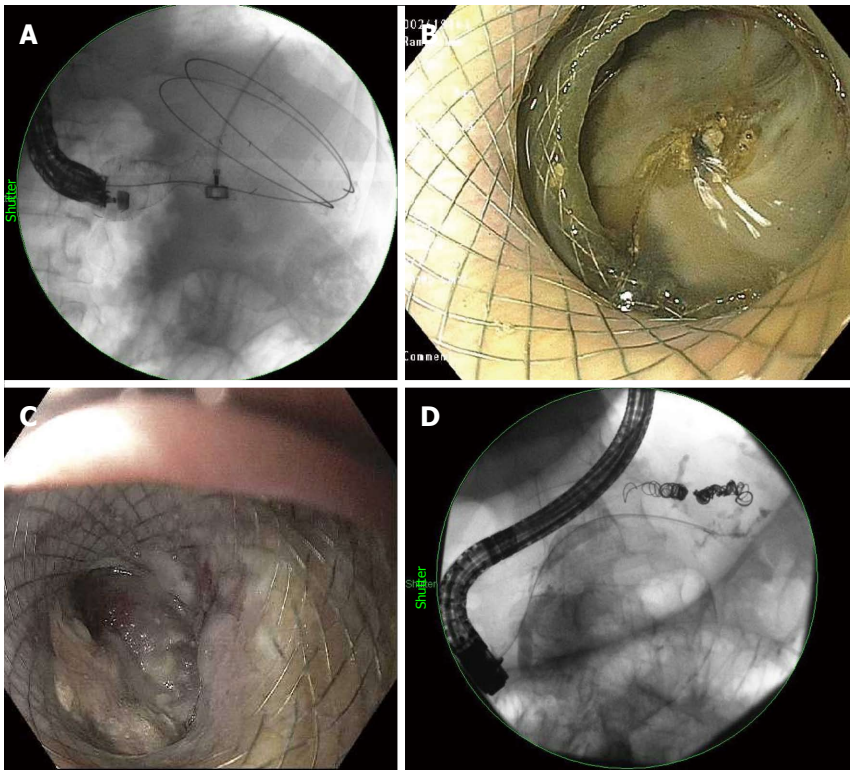

Figure 6 Fluoroscopic visualization of an esophageal fully-covered self-expanding metal stents deployed into walled-off necrosis (A), a pancreatic duct leak (D), endoscopic visualization of a lumen-apposing metal stent deployed into walled-off necrosis (B, C).

success rate in 52 patients, with a 5.4% complication rate. Additional multi-center studies are needed, but LAMS represent a promising advance in the endoscopic management of WOPN (Figure 6B and C).

Cumulatively, these studies illustrate that while endoscopic necrosectomy is efficacious, it is a complicated procedure requiring a high-level of skill in endoscopy with complications occurring even in the most experienced of hands and requiring the presence of a strong multi-disciplinary team to be successful. The incorporation of metal stents that allow for a large drainage lumen and the advancement of an endoscope through the stent lumen for DEN is a major advance, which may ultimately improve efficacy and decrease complications associated with these procedures (Table 1)[99-102].

ENDOSCOPY *VS* PERCUTAENOUS OR SURGICAL DRAINAGE

A recent randomized multicenter trial from 2012 directly compared endoscopic necrosectomy and surgical necrosectomy (video-assisted retroperitoneal debridement with open laparoscopic necrosectomy for rescue) in 22 patients[103]. Their results showed that endoscopic therapy was associated with a lower post-procedure inflammatory response (as demonstrated by interleukin levels), a lower complication rate, fewer pancreatic fistulae developments, and less pancreatic enzyme use on 6 mo follow-up. Amore recent study from 2014 directly compared a step-up approach starting with percutaneous drainage and escalating to more invasive therapy as needed to DEN in 24 patients[104]. Their results demonstrated a resolution rate of 92% *vs* 25% in the necrosectomy *vs* percutaneous drainage group, with 9 of 12 patients requiring surgery after percutaneous drainage alone. Additionally, less antibiotic use, pancreatic insufficiency, and hospitalization was seen in the endoscopic necrosectomy group.

ERCP FOR PANCREATIC DUCT EXPLORATION

An important component in the management of PFCs is ensuring the integrity of the pancreatic duct (PD) *via* ERCP. Disruptions in the PD are associated with an increased severity of pancreatitis, an increased risk of recurrent attacks of pancreatitis and long-term complications, and a decreased rate of PFC resolution after drainage[105-110] (Figure 6D).

PD DISRUTPION AND SEVERITY OF PANCREATITIS

A PD disruption has been shown to be associated with a more severe course of pancreatitis. A retrospective review of 105 patients with acute pancreatitis found that nearly half of patients with severe pancreatitis

had concurrent PD disruption, while a normal PD was noted in 100% of patients with mild pancreatitis[105]. Similarly, in a retrospective review of 144 patients with severe pancreatitis, Lau *et al*[109] found that patients with a PD leak were 3.4 times more likely to have pancreatic necrosis. Thus, assessing for a PD disruption in patients with pancreatitis is an important prognosticating step.

PD DISRUPTION AND RECURRENT PANCREATITIS/LONG-TERM COMPLICATIONS

In addition to predicting the severity of pancreatitis, a PD disruption can also predict the likelihood of long-term complications and recurrent episodes of pancreatitis. Howard *et al*[106] looked at 14 patients with WOPN who developed recurrent pancreatitis after initially-successful debridement, and found that all 14 patients had a pancreatic duct abnormality on either ERCP or MRCP. No other predictive factor of recurrence was identified. Nealon *et al*[107] demonstrated that in 174 patients with severe pancreatitis, long-term complications such as sepsis and recurrent pancreatitis occurred in 36%-38% *vs* 0% and 62%-89% *vs* 7% of patients with an abnormal PD compared to those with a normal PD.

PD DISRUPTION AND PFC RESOLUTION

Assessing for PD disruptions can also predict treatment success. In the same study as above-mentioned, Nealon *et al*[107] demonstrated that altered PD anatomy is directly correlated with a decreased rate of pseudocyst resolution. In 563 patients with pseudocysts, they found that spontaneous resolution occurred only in 0%-5% of patients with a ductal disruption compared to 87% of patients with a normal pancreatic duct. Similarly, Trevino *et al*[108] demonstrated improved PFC resolution of both pseudocysts and WOPN in patients who underwent PFC drainage with transpapillary PD stenting compared with PFC drainage alone (97.5% *vs* 80%). Of note, undergoing ERCP was not associated with any increase in mortality, the need for necrosectomy, or hospital length of stay.

CONCLUSION

Pancreatitis can frequently result in the development of fluid collections, ranging from simple pseudocysts to WOPN. The initial step in management of these collections is ensuring adequate nutritional support is provided. Enteral nutrition is preferred over parenteral nutrition, with post-pancreatic jejunal feeding being the optimal enteral route in patients with moderate or severe disease. Symptomatic PFCs require drainage. Endoscopic drainage can be successfully accomplished with improved safety and efficacy as compared to surgical or radiologic approaches. Furthermore, patients with WOPN can safely undergo endoscopic necrosectomy, obviating the need for surgical exploration. Lastly, in all patients with suspected PD disruption, ERCP with PD exploration should be performed and if MRCP is available, it should be used accordingly to rule out pancreatic duct disruption in low probability patients.

In summary, all forms of PFC can be safely and effectively managed by a variety of endoscopic procedures.

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REVIEW

Hepatitis E virus: An ancient hidden enemy in Latin America

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Abstract

Hepatitis E virus (HEV) infection is a common cause of acute clinical hepatitis worldwide. HEV is an RNA-containing virus and the only member of the genus *Hepevirus* in the family *Hepeviridae*. Human HEV is classified into four genotypes widely distributed across the world. The virus is mainly transmitted *via* the fecal-oral route, and water-borne epidemics have become characteristic of hepatitis E in developing countries, including those in Latin America. The zoonotic potential of HEV is broadly recognized. Thus, there is an urgent need to re-evaluate virus transmission scenarios and to enforce epidemiological surveillance systems. Additionally, it is known that HEV infections, initially defined as self-limiting, can also take chronic courses in immunocompromised patients. Moreover, we recently reported a high seroprevalence of HEV in samples from cirrhotic patients with no other etiological agents present, suggesting the potential role of HEV in the development of chronic liver illness. In this review, HEV genomic variability, transmission, chronic infectious course, zoonotic potential and treatment are discussed. Focus is placed on the impact of HEV infection in Latin America, to support the development of specific control strategies and the handling of this important and typically imperceptible viral infection.

Key words: Emerging diseases; Zoonosis; Viral genotypes; Mexico; Hepatitis E virus-chronic infection

Core tip: Despite the widespread presence of hepatitis E virus (HEV), this pathogen is not commonly considered from a global public health perspective. Active research on hepatitis E both in animals and humans has provided novel insight into HEV pathogenesis, zoonotic potential and its role in chronic liver disease. Detailed guidelines for tracking cases need to be developed to contain the virus. This action is particularly necessary in endemic and emerging situations in regions with a higher risk of developing the infection, including Latin America.

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INTRODUCTION

Multiple outbreaks of viral hepatitis have been documented around the world since ancient times. In Latin America, viral hepatitis may have been implicated in the extermination of more than half the population of Mesoamerica in colonial times[1]. In the past century, hepatitis A virus (HAV) and hepatitis B virus (HBV), followed by the more recent description of hepatitis C virus (HCV), have been identified as causative agents of viral hepatitis. Hepatitis E virus (HEV), although probably also ancient, was not recognized as a new virus-causing hepatic disease until 1980[2], which is the main reason for the limited information on this viral disease.

HEV infection is responsible for the half of all outbreaks of acute liver disease in endemic areas[3]. HEV, which was formerly known as non-A, non-B hepatitis-causing infectious virus, is mainly transmitted *via* the fecal-oral route through contaminated water and is primarily found in areas with inadequate sanitary conditions. Other routes, such as organ transplants and zoonotic transmission, may possibly play important roles in transmission. Although the disease is usually associated with rather low fatality and mortality rates (0.2%-3.0%), in some highly susceptible populations the estimated infection rates have been reported in the range 15%-20%[4]. Of interest, in HEV-positive pregnant women, mortality due to fulminant hepatic failure has been reported to reach up to 25% in infected individuals[4,5], which is the highest value reported so far for HEV-caused fatalities.

HEV infection and HEV-associated diseases represent a major public health problem. It is estimated that 2.3 billion people are infected globally. HEV is responsible for nearly 50% of acute viral hepatitis in developing countries in Asia, Africa and Latin America[6]. Acute infections mostly affect adults, 15 to 40 years of age and are symptomatically mild. Studies from Asian endemic regions indicate a high seroprevalence, with rates ranging from 15% to 60%[3]. Chronic disease related to HEV has been reported in immune-suppressed individuals, such as organ transplant recipients, patients receiving chemotherapy, and HIV-infected patients[3,6], in whom chronic HEV infection may lead to the development of hepatic fibrosis and cirrhosis[7].

HEV, the etiological agent of hepatitis E disease, has been classified into at least four genotypes and several subtypes (Table 1). HEV genotypes 1 and 2 are hyper-endemic in Asia and Africa, and frequently cause outbreaks of acute hepatitis. HEV genotype 3 is prevalent in developed nations, where sporadic acute hepatitis has been reported[3,6]. HEV genotype 4 is almost exclusive to Asia, and it is recognized as the most frequent cause of the sporadic hepatitis E cases that affecting humans in China[8].

Initially considered an infection associated with the use of low-quality water sources, its importance has increased due to animal reservoirs. In the last decade, HEV has gained increasing attention as depicted in Figure 1. The detection of HEV in animals raised concerns about the risk of zoonotic transmission to humans. Thus, the need to involve animal health into

Table 1 Hepatitis E virus genotypes reported for Latin America

Genotype	Region	Species[1]			Detection method/input specimen[2]	
		Humans	Swine	Other[3]	RT-PCR	Serum
Genotype 1	Uruguay	X			X	X
	Venezuela	X			X	
	Cuba	X				X
	Mexico	X			X	X
Genotype 2	Mexico	X	X		X	X
Genotype 3	Argentina	X	X	X	X	X
	Brazil	X	X	X	X	X
	Bolivia	X				X
	Cuba	X			X	
	Venezuela	X			X	
	México		X	X	X	
	Uruguay	X			X	
	Chile	X			X	X
	Costa Rica		X		X	
Genotype 4	China, Japan, Taiwan (not in Latin America)	X	X		X	X

[1]According to reports found in PubMed, indicates infection regardless of disease condition of the host (with or without clinical signs). [2]Origin of the host in analyzed samples. Reverse Transcription PCR (RT-PCR) was the method used to detect genomic RNA of HEV. When serum was the input material, the detection of anti-HEV specific antibodies (seroconversion) was indicative of current contact (active infection) or past infection. [3]Host or developed in chickens, deer, rodent and other mammals.

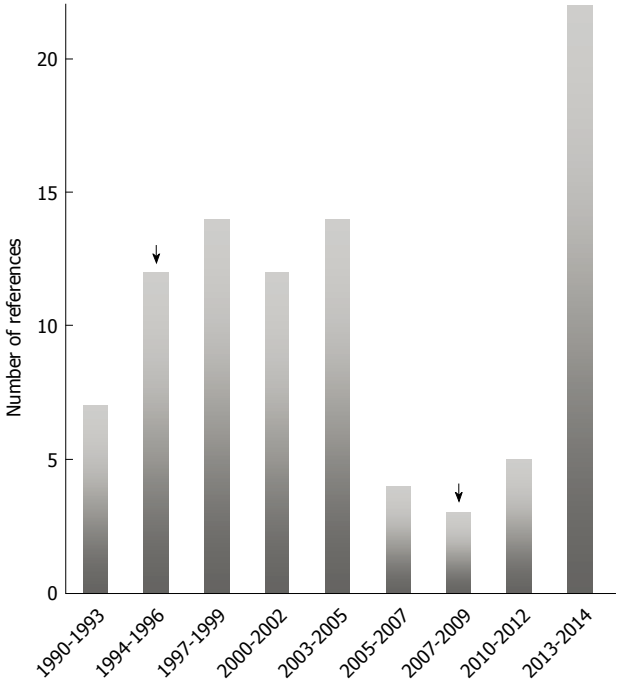

Figure 1 Timeline of the number of papers about hepatitis E virus. Based on references found in PubMed when hepatitis E virus (HEV) was used as a search keyword. All the years prior to 1990 clustered at first time point. Awareness was more prominent following the demonstration of chronic hepatitis as caused by HEV. Arrows highlights first report of zoonotic transmission (left arrow), and the demonstration of chronicity due to HEV infection (right arrow).

a single approach was recently recognized as the One Health initiative stated of the World Health Organization (WHO), which was developed to cope with such multi-source causes of disease. Human HEV outbreaks have been reported in Latin America, and specifically, twice in Mexico[2]. In both reported cases, the viral genotype 2 was detected, and this remains the only viral variant from this country[2]. HEV has also been detected in pigs from Mexico and Costa Rica[6]. Moreover, recent reports demonstrated that HEV circulates in Uruguay[6] and central Argentina[9]. Additionally, it is endemic in the Brazilian Amazon[10] and is postulated to be present in Bolivia based on the detection of antibodies against HEV[11]. Based on the limited knowledge available about the distribution of HEV viral variants in Latin America, in this review a general scientific landscape is presented to update HEV epidemiology literature and to assess the epidemiological risk for humans in this region.

VIRUS CAUSING HEV DISEASE

Hepatitis E disease is due to infection with HEV virus, a member of the *Hepaviridae* family[3]. This family includes the genus *hepevirus*, which contains viruses isolated from humans, avian species, mice, rats, and several other mammals such as boars, rabbits, camels, goats, ferrets, and mongooses. The viral particle is a non-enveloped sphere with approximately 32-34 nm in diameter. The viral genome consists of a single-stranded, positive-sense RNA molecule 7.2 kb in size. The genome organization includes a 7-methylguanosine cap structure attached to the 5′ end, a noncoding region in 5′ end, an open reading frame 1 (ORF1) and two other ORF2 and ORF3, which are partially overlapping. HEV RNA also has a non-translated region at the 3′ region spanning approximately 27-35 nucleotides (nt), and the 3′ end is extended by a poly A tract[5] (Figure 2).

The first genomic region, ORF-1, gives rise to a protein of approximately 1693 amino acids (aa) that encodes non-structural proteins and enzymes involved in viral replication, transcription and protein processing[12]. The most downstream region, ORF-2, consists of 1980 nt, ending with 65 nt before a poly-A tail signal, which encodes a 660-aa, a glycosylated protein corresponding to the structural protein of the capsid[3,6]. The ORF-2 protein contains epitopes that induce the neutralizing antibodies located mainly near the carboxyl end. The intermediate region, ORF-3, overlaps with a single nt at its 5′ end with ORF-1 and with 328 nt with ORF-2. ORF-3 encodes for 123 aa, a small, approximately 13.5 kDa protein, that is phosphorylated[5]. Also known as phosphopeptide, this small protein was reported to be associated with the hepatocellular cytoskeleton and forms a complex with the major capsid protein ORF-2, suggesting their involvement in virion assembly. ORF-3 might also have regulatory functions involved in the modulation of cellular signaling transduction[5,13]. Additionally, the ORF-3 protein is thought to contain neutralizing epitopes toward its 3′ end[12,13], which highlights its importance during the viral infection cycle.

HEV SEROEPIDEMIOLOGY IN LATIN AMERICA

Because HEV is primarily transmitted *via* the fecal-oral route, most outbreaks have been described as originating from a source of water. This situation occurs mainly in developing countries with a temperate climate, high population density and poor sanitary conditions[4]. Since the first epidemics described in New Delhi, India (1955-1956), many others have occurred in India (Kashmir), Nepal (Kathmandu) and China (Xinjiang Province, 1986-1988)[3]. In Latin America, the only major outbreak of HEV occurred in Mexico from 1986-1987[2,14].

The total prevalence of antibodies against HEV in endemic countries is variable (3%-27%)[4]. In contrast to other enteric viruses, such as polio or HAV, the prevalence of IgG anti-HEV is lower in children and young people than it is in adults[2]. In non-endemic areas with proper sanitary conditions and a well-controlled water supply, the prevalence of antibodies against HEV in the general population is relatively high (up to 7%-10%), and is even higher in certain endemic areas[2]. In Mexico, a study analyzing the

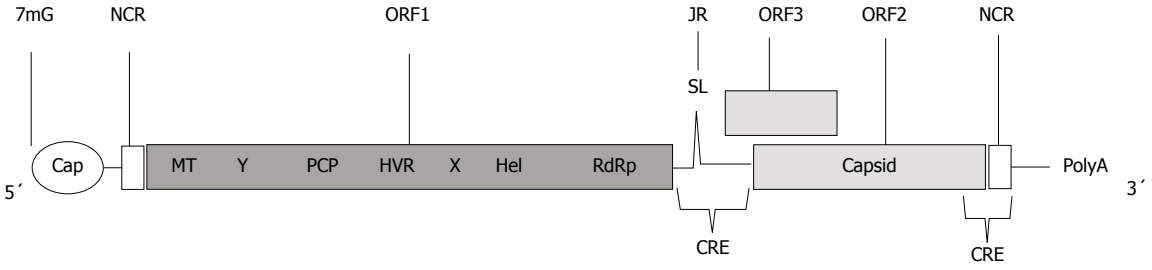

Figure 2 A schematic representation of the RNA- hepatitis E virus genome. RNA encodes for multiple proteins from three Open Reading Frames (ORFs). ORF1 comprises the methyltransferase (MT), Y domain, a papain-like cysteine protease (PCP), hypervariable region (HVR), X macrodomain, helicase (Hel), and RNA-dependent RNA polymerase (RdRp). ORF 2 encodes the capsid protein, and ORF3 encodes a cytoskeleton-associated phosphoprotein. 5´Cap and 3´PolyA modifications are also depicted. The junction region (JR), stem-loop structure (SL), and cis-reactive element (CRE) have been described elsewhere[12].

serum samples of 3549 individuals found a HEV seroprevalence of 10.5% in young adults and children of different socioeconomic status and from various regions within the country. This seroprevalence increased with age, from 1.1% in children under five years old up to 14% in people between 26 to 29 years of age[14,15]. Age, the type of community, and the level of education have been described as risk factors for infection[2]. A seroprevalence of 6.3% with a clear predominance of men older than 50 years was then confirmed in the same Mexican study[15]. In Mexico, HEV has also been detected as a cause of disease in the State of Hidalgo[16], and circulates in pig populations[17]. So far, no studies have evaluated HEV zoonotic potential or the risk of transmission in the food chain in Mexico.

From recent studies, it seems that HEV has been found in several other regions in Northern Mexico. Analysis of blood samples from 557 low-income, pregnant women in El Paso, Texas (United States) and 307 women in Ciudad Juarez showed a seroprevalence for HEV of 0.4% and 1.6%, respectively[18]. Additionally, a study of 273 serum samples from rural adults in Durango state revealed a higher (36.6%) seroprevalence than that reported in other Mexican regions[19]. This same study reported serological evidence of HEV exposure in 150 Mennonites in the same geographical area. However, Mennonites had a lower seroprevalence (6.7%) of HEV antibodies than the general rural population (40.7%) and, as reported for other groups, the seroprevalence in Mennonites increased with age[20]. The findings of a higher prevalence in older age subjects could represent a latent infectious stage in which the virus circulates in a subclinical form, or the constant permanence within an undetected animal reservoir.

An evaluation of HEV presence in higher risk population has been conducted in Mexico. The study of 439 pregnant women in Durango, Mexico revealed an HEV seroprevalence of 5.7% and an association between unpasteurized cow milk consumption and HEV exposure[21]. In Latin America, HEV has also been found in Brazil[22,23], Chile[24], Argentina[9], Costa Rica[25], Bolivia[6,11] and Uruguay[26] (Table 1). Currently, it is accepted that in these regions, HEV has a high prevalence.

HEV GENOMIC VARIABILITY: THE CASE OF LATIN AMERICA

As an RNA virus, HEV has been recognized to exhibit broad genetic diversity although no serotype variation has been identified so far. A single serotype has been described and is composed of at least four different genotypes differing in geographic distribution and host (Table 1). Studies characterizing the genome of HEV from the same outbreak have found sequence similarities in the range of 95%-98% at the nucleotide level and 95%-100% at the amino acid level[27,28]. Support for the quasispecies nature of HEV relies on this reported genetic heterogeneity, but its impact on epidemiology has not been assessed. The genomic sequence dissimilarity has been proposed to extend the classification to incorporate HEV variants detected in many animal species, but the virus that infects humans belongs to the same four genotypes previously recognized[29]. Two additional genotypes have been proposed following the detection of HEV in wild boar in Japan, and the taxonomy of Hepeviridae is currently under revision[30]. At present, HEV is still classified into the same four genotypes, which were further subdivided into subtypes[29]. Most viral diversity relies on RNA, and alternative protein sequence-based methods could be useful for this multi-host pathogen (see Table 1) to avoid multiple subdivisions and fragmentation of this single-serotype virus. Notably, the availability of a detailed description of viral epitopes[30] could permit the use of immune-oriented methods to understand current and upcoming sequence diversity.

HEV genotypes 1 and 2 have been isolated from humans and associated with HEV outbreaks[5]. Genotype 1 is considered endemic in some areas of Asia and Africa and was also detected in Cuba and Venezuela[6,27]. In Uruguay, an indigenous HEV, genotype 1, was recently described[26]. Genotype 2 has been reported in Mexico and some local variants from Africa[2,6,27]. HEV genotypes 3 and 4 were initially

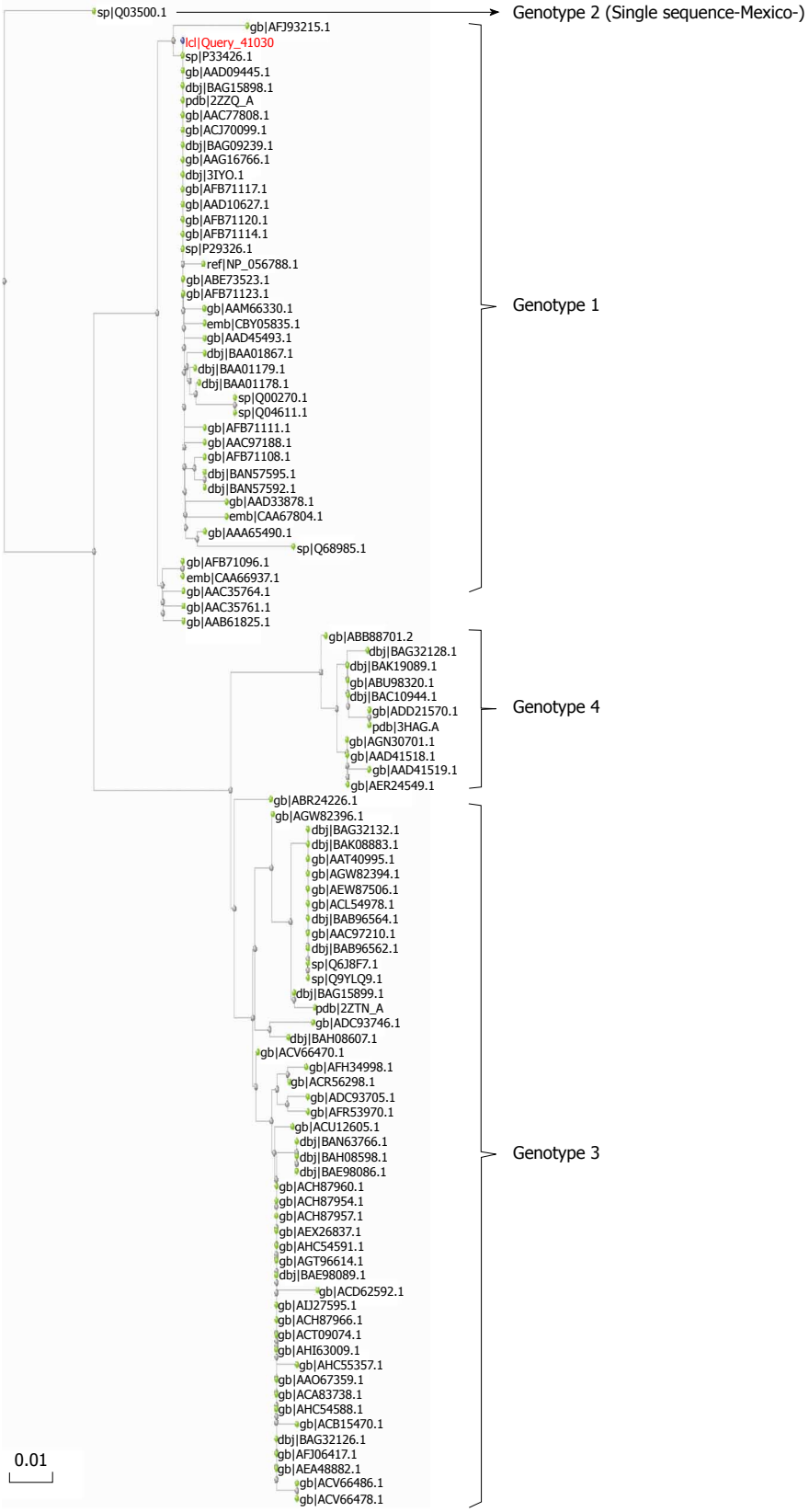

Figure 3 A phylogenetic tree for hepatitis E virus according to their amino-acid sequences from the recognition domain E2 (E domain, residues 455 to 606, based on US-1 strain) in the open reading frame capsid protein. This region corresponds with the binding domain of neutralizing Mab 8C11[32,33]. It contains all epitopes demonstrated to elicit neutralizing antibodies. This region is sufficient to provide protection, and is the basis of the current recombinant vaccine Hecolin 293 approved in China. The analysis was based on 100 sequences representing the diversity of HEV sequences. The tree was constructed by the Maximum Likelihood method using PhyML software, as implemented in server http://phylogeny.lirmm.fr[33]. The scale bar represents amino-acid substitutions and the statistical support was obtained from 100 Bootstrap replicates.

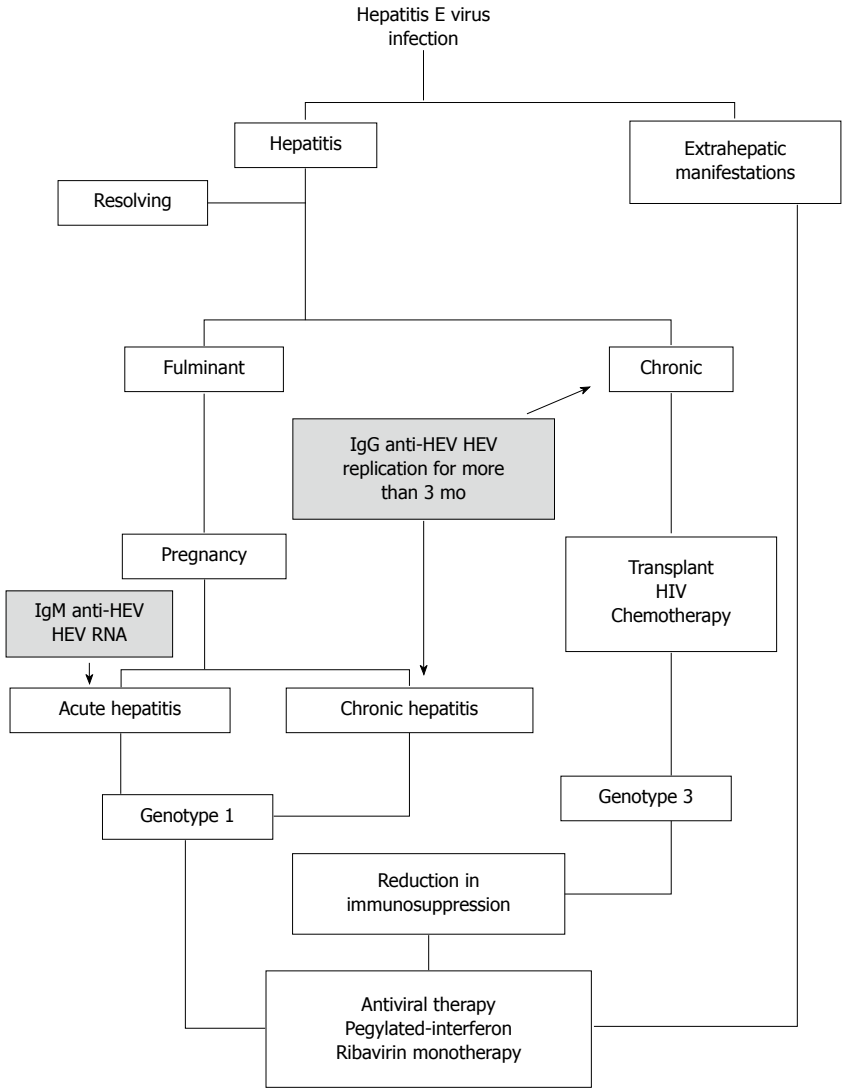

Figure 4 Patterns of hepatitis E virus infection. The typical clinical courses taken by hepatitis E virus (HEV) infections are depicted. The scheme is based on the clinical approach followed at different institutions, as first revised in Kamar *et al*[13]. The shaded text corresponds to the clinical laboratory markers used to classify the degree of disease caused. This model is based mainly on infections with HEV from genotypes 1 and 3, as shown. Fewer data are available for genotypes 2 and 4, but the same general model may apply.

isolated after causing infection in humans and animals, mainly pigs. In humans, these genotypes 3 and 4 have been associated with acute hepatitis sporadically during outbreaks[10]. HEV genotype 3 includes isolates from non-endemic regions (the United States, Italy and Greece), and its occurrence is considered to be related to traveling to endemic regions, representing a group of more diverse sequences[4-6,10]. HEV genotype 4 is found in Asia, and is particularly endemic in China, and Central Europe[3,4]. HEV genotype 3 is the most frequent genotype found in South America, where the HEV epidemiology appears to be composed of at least three of the four known genotypes[3,4,6] (Table 1). It is interesting that the clinical manifestations of HEV have been associated with distinct ages depending on the infectious genotype. In the Indian outbreak, which primarily consisted of genotype 1, the age group with the highest incidence of infection was between 20 and 29 years. Characterization by extensive sequence analysis of three regions of HEV genome from Uruguay revealed that these viruses were closely related to a set of European strains[26] and, thus, dissimilar to Brazilian, Argentinean and Bolivian isolates[6]. The outbreaks reported in Mexico and those associated with genotypes 1 and 2 in developing countries have shown the highest incidence among individuals in the same age range. In contrast, sporadic infections in industrialized countries have been associated with genotypes 3 and 4, with an average age of highest incidence of 60 years. HEV detected from Argentina and Brazil are more related to viruses from industrialized countries (North America and Europe), whereas the HEV in the Caribbean and Mexico include viral genotypes more closely related to outbreaks in Africa and Asia[2,6]. The heterogeneity of HEV viruses in Latin America can be demonstrated by the

phylogenetic analysis of their sequences as detailed in Figure 3[28,29], and particularly according to their amino-acid sequence from recognition of the E2 domain in ORF2 that corresponds to the major recognition epitope for neutralizing antibodies[30-32]. The prevalence observed in this region and its genetic heterogeneity highlight the urgent need to improve our knowledge of the molecular epidemiology of HEV in Latin America. Thus, single or multiple re-introductions of HEV may be detected to prevent further outbreaks.

TRANSMISSION

HEV is primarily transmitted *via* the fecal-oral route. As such, the epidemics of hepatitis E in endemic areas are due to contaminated water[2]. The optimal circumstances for hepatitis E epidemics arise when untreated wastewater comes into contact with drinking water during times of heavy rain. Examples of these circumstances included the 1950s epidemic in Delhi (India), which was preceded by heavy rains and floods[6], and outbreaks in Mexico, which have coincided with the rainy season. There is a direct correlation between a high incidence of positive serology of HEV and the use of superficial waters without boiling for food consumption or personal hygiene[6]. Some studies have described the presence of viral particles and RNA in the wastewater of cities after the slaughter of swine in industrialized countries[33,34]. However, subsequent studies have not confirmed any risk of HEV infection *via* this type of water contamination in industrialized countries[35]. This discrepancy may reflect the difficulty of detecting pathogens in water samples, particularly in the case of HEV RNA, which is very labile. Clearly, there is still a great need for systematic studies that identify the risk represented by such water conditions in Latin America because many areas are considered to have poor health conditions and deficient sanitary treatment of the potable water systems.

The incidence of HEV transmission from person to person is low, representing between 1 and 2% of the total number of cases described[5]. In contrast, the rate of HEV transmission from mother to child, though variable, is quite high (30% to 100%)[20,21]. Up to two-thirds of pregnant women infected with HEV may have premature births and high neonatal mortality[36]. Reports of HEV RNA in the blood of newborns, when the virus could not be detected in the mothers, have also been published as a possible case of vertical transmission[37]. Additionally, the presence of HEV in multiple animal reservoirs shows that the origin and transmission of HEV disease are in need of urgent revision. Thus, animal reservoirs used as a food source cannot be discarded as risk factors for spreading HEV[13].

PATHOGENESIS

After entering the body, HEV virus shows exquisite tropism for the liver, *via* as-yet-unknown mechanisms. The virus accumulates in the bile, reaches the intestine through the bile duct, and then can be found in the feces after approximately two weeks of infection. During the first two weeks, anti-virus specific antibodies can be found in the serum[4,5]. HEV has been reported in the blood or feces for longer periods, up to 16 wk, depending on unknown conditions. The more prolonged maintenance of HEV in asymptomatic individuals may result in reservoirs during inter-epidemic stages over time, resulting in sporadic infections among persons exposed to contaminated food or water[4]. HEV is shed in the feces of infected individuals as infectious, nonenveloped virions with their genome encapsidated in a naked protein shell[10,13]. However, recent reports suggest that HEV circulates during acute infection in a membrane-wrapped form in which the encapsidated RNA is completely enveloped and sequestered from neutralizing antibodies, and yet has an infectivity equivalent to that of naked virus particles. These membrane-wrapped forms of HEV differ from classical enveloped viruses in that the surrounding lipid bilayer appears to be devoid of virally encoded proteins[13]. This would allow the extracellular virus to masquerade as a host-derived exocytic vesicle and likely facilitates its dissemination within the host, but this hypothesis remains to be tested.

During the infection cycle, typical humoral responses have been described with less information available for the corresponding cellular component of the immune response. The role of the host response in the self-damage to liver cells is poorly understood, and there is agreement regarding the prominent role of the immune system plays in the resultant liver damage[3]. The identification of specific IgM antibodies for HEV in the serum coincides with the appearance of symptoms. HEV-specific IgG antibodies can be detected shortly after the emergence of IgM antibodies, and IgG values increase during the acute phase of the disease and remain detectable in the serum for several years[3,4].

The lack of a cellular or animal model hampers the progress of studies regarding the pathogenesis of HEV virus. Attempts to culture the virus have given rise to a few reports on cells lines permissive for HEV replication while in culture[10,13]. However, no standard methodology for viral isolation has been established, and the characterization of infection is performed using only serological and molecular methodologies (Table 1). The understanding of viral pathogenesis is in urgent

need for *in vitro* methods and *in vivo* approaches that might evoke the viral life cycle.

HEV-chronic infection in immunocompromised patients

Although HEV infections are defined as acute and self-limiting, we previously reported a high seroprevalence of HEV in samples from cirrhotic patients with no other etiological agents, suggesting the potential role of HEV in the development of chronic liver illness[2]. In fact, it has been recently accepted that HEV infections may take chronic courses under specific circumstances, such as in immunocompromised individuals. Although chronic HEV infections have been mainly diagnosed in the organ transplanted population[38-43], chronic HEV infections have also been found in patients receiving chemotherapy[44] and in patients coinfected with HIV[45,46].

HEV and transplants

The HEV seroprevalence is high in liver transplant recipients[38]. The prevalence of HEV infection in organ transplant patients (OT) ranges from 2.3% to 43.9%, depending on the serological test employed[47-53]. The incidence of HEV RNA in OT with increased liver enzyme levels ranges between 4.3% and 6.5%[38,53]. To date, HEV is considered to cause chronic infection with rapidly progressive cirrhosis within 1-2 years of infection in organ-transplanted patients[53,54], faster than reported in HCV-infected OT. This rapid progression is found in liver and non-liver transplant patients[54]. The administration of immunosuppressive medication to prevent organ rejection appears to be an important risk factor for developing a chronic infection caused by HEV. In particular, tacrolimus, a potent immunosuppressant, has been associated with chronic HEV infection[55-57]. Advances in the description of optimal immunosuppressive protocols for HEV-infected patients are currently in progress[58,59].

A diagnosis of chronic hepatitis infection is defined by the presence of genomic viral content for more than six months. However, in the setting of organ transplantation, it has been found that no spontaneous clearance of HEV occurs between 3 and 6 mo after an acute infection[13]. Thus, chronic HEV infection should be considered when HEV replication persists for more than 3 mo. Moreover, given the liver enzyme abnormalities often attributed to hepatic graft-versus-host disease or drug-induced liver injury in liver transplant recipients, the misdiagnosis of HEV is frequent. Symptoms are present in only 32% of OT patients, with fatigue being the main symptom and, in contrast to acute hepatitis A virus infection, clinically apparent jaundice is uncommon. Liver enzyme levels are increased (300 IU/L) but are lower than those observed in immunocompetent patients (1000 to 3000 IU/L)[57]. Considering that HEV infection may occur in donors, this emphasizes the need for HEV screening not only after transplantation but also in donors presenting liver function abnormalities[60]. HEV diagnosis in OT with elevated liver enzymes is advised and is based on HEV RNA testing as antibody assays are typically not sensitive enough. HEV seroconversion may be delayed and may not occur in some patients. Thus, the molecular detection of HEV RNA is essential to exclude an HEV infection in patients who are negative for anti-HEV IgM and to assess the evolution of infection. To date, no cases of chronic HEV genotypes 1, 2 or 4 have been reported. All chronic cases have been associated with HEV genotype 3. Interestingly, fulminant hepatitis in pregnant women has been related to viral genotype 1[13]. Thus, the determination of genotypes may be crucial to predicting the disease outcome.

HEV, chemotherapy, and HIV co-infection

The systematic analysis of the incidence of chronic HEV infection among hematological patients receiving chemotherapy has not been conducted. Among the small number of reported cases are patients treated for lymphoma, chronic myelomonocytic leukemia, and B-cell chronic lymphocytic leukemia[59-62]. The incidence and HEV seroprevalence among stem cell transplantation patients is in progress. Preliminary, evidence from two independent studies report an anti-HEV seroprevalence of 36% and 5.6% among cell transplanted patients. However, in both studies, ongoing HEV infection was absent[63,64]. This piece of information has induced clinicians to screen patients in chemotherapy with abnormal function tests for HEV RNA. Similarly, chronic infection with HEV has been described in individuals with HIV infection. Based on the limited number of analyzed cases, the seroprevalence of anti-HEV IgG in HIV-positive patients ranges from 1.5% to 11.2%[65-75]. However, the incidence of HEV infection defined by the presence of HEV RNA in the serum is low, ranging from 0 to 1.3%[66,70-75].

A small number of cases of HEV coinfection confirmed by the detection of genomic viral content have been documented around the world[45,66-68,71,73,76-78]. A high HEV seroprevalence in HIV-infected patients has been documented in both HIV-infected patients with unexplained liver disease[79] and HIV-infected patients in the absence of chronic liver infection[80]. As detailed above, genotype 3 has been the HEV variant detected among HEV-related cirrhosis cases in HIV co-infected patients[67,68,70,81]. The clinical presentation in patients receiving chemotherapy and in the HIV co-infected patients is similar to that found in OT patients. Moreover, HEV infection can even result in extra-hepatic manifestations both during and after the resolution of infection[80]. However, data regarding the epidemiology of hepatitis E in particular populations is limited. Further studies are required to determine the exact role of HEV in the development of liver damage in immunocompromised and immunocompetent indi-

viduals.

ZOONOSIS

HEV is widely recognized as a zoonotic infectious agent[82-84]. Recently, a re-visited American population had a 6% seroprevalence of anti-HEV antibodies in the general population. For this US-based study, Hispanic race, and "meat" consumption were identified as factors associated with HEV-seropositivity. No significant association was observed with low socioeconomic status, water source, or level of education. In the multivariate analysis, only older age remained predictive of HEV seropositivity[81,84]. Of note, in previous studies in the United States, having a pet in the home was identified as another important factor for HEV-seropositivity[83,84]. From studies in other countries, it seems that the presence of anti-HEV antibodies in pet dogs could be approximately 1%, and this factor could also become another component to understanding the epidemiology of HEV[85,86].

In the 1990s, the presence of antibodies against HEV in swine was described for the first time[84,86] (Figure 1). Subsequently, the experimental infection of swine with swine HEV or isolates of human origin demonstrated that infected animals exhibited viremia, releasing the virus along with their feces while showing no evidence of clinical or biochemical disease. Studies in America, Asia, and Oceania, have shown very high prevalence rates, ranging between 20% and 100%[86]. In Latin America, HEV circulates in animal species, including swine, as reported in Brazil[87] and Argentina[88]. A study analyzing the presence of antibodies against swine HEV in Mexico and Thailand found positivity for IgG anti-HEV in 81% of the swine analyzed in Mexico[6,17]. The sequence analysis of a total of 44 positive samples isolated from Mexico and Thailand were genotype 3, supporting the notion that only genotypes 3 and 4 have zoonotic potential[17,89-94]. This finding is of particular interest as it considers the genotypes found in industrialized countries, suggesting that the mechanism of infection in these regions could be zoonotic. Moreover, the study of 87 livers collected from pigs destined for human consumption in Nuevo Leon, Mexico revealed that between 19.54% and 22.5% of the livers were positive for genomic HEV[89]. These findings indicate that HEV-infected meat may constitute a source of contamination[82,89]. The initial suggestion that HEV could potentially be a zoonotic agent has been supported by data showing a high prevalence of anti-HEV antibodies in farms in both endemic and non-endemic areas[82]. Moreover, swine isolates are genetically more related to human variants in the same geographical region than to swine strains from other parts of the world, which supports the zoonotic nature of transmission. Furthermore, the described cases of acute hepatitis after ingesting raw meat from HEV-infected deer demonstrate the potential zoonotic characteristics of HEV[90,91].

In addition to swine, HEV infections have been detected in other animal species. Anti-HEV antibodies and the HEV RNA genome have been detected in animals such as cattle, sheep, dog, deer, cat, goat and nonhuman primates[82,90]. The presence of specific antibodies only (seroprevalence) has been described by several studies in chickens, dogs, cattle, cats and rodents[82,94-103]. In most cases detecting and analyzing HEV genome, sequencing results obtained from a particular location have been very similar regardless of the species of origin. Altogether, these observations confirm of the zoonotic circulation of HEV virus crossing the species barrier frequently[84,96].

Experimentally, a rabbit HEV strain was successfully demonstrated to be transmitted to pigs, indicative of the ability of HEV to cross the species barrier[91]. The zoonotic transmission of HEV from deer[90] and pet cats[95] to humans has also been reported[82,84]. In addition, it has been demonstrated that avian HEV, although genetically less related to human HEV compared to swine, shares antigenic epitopes with both[98]. At least three genotypes of HEV have been documented from chickens worldwide[92-94], and these are different from the genotypes described for mammals. HEV infection in chickens is enzootic and affects 71% of chicken flocks in the United States. HEV infection in chickens is mostly subclinical, although 30% of chickens were seropositive for avian HEV antibodies[92,94]. While the evidence of avian infection in humans is currently lacking[93], the spread of bird-to-bird infection has been reported[94]. HEV has been isolated from a variety of animal species ranging from bats, chicken, camel, cutthroat trout, deer, ferret, fox, mink, mongoose, moose, pigs, rabbits, rats and wild boar[91-96].

The high incidence of HEV infection in Mexican swine requires adequate epidemiological surveillance in high-risk groups near farms. Also, although some studies have revealed that domestic pet veterinarians are at no increased risk of hepatitis E compared to the general population[83], the data have primarily come from Asia and Europe. Specific studies in Latin American countries are required to determine the potential risk of infection in this group. Descriptions of genotypes in rural areas with poor sanitary conditions are needed to provide the necessary data for controlling these infections, the incidence of which at present has been underestimated.

TREATMENT

There is no specific prophylactic treatment for acute hepatitis E infection. The results obtained in animal models suggest that the use of specific Ig, when faced with high HEV titers, can be useful during outbreaks[3]. The existence of a single serotype of HEV supports the possibility of generating a vaccine that has broad cross-reactivity against all known genotypes. The availability of such vaccine would be useful to protect against HEV

infections and to prevent local outbreaks in developing countries, especially in immunosuppressed individuals, inhabitants of endemic regions and travelers to endemic areas. In 2011, the first vaccine against HEV (Hecolin, based on strain HEV-239), was licensed in China[97]. This vaccine is not yet available in the rest of the world. In the absence of a vaccine, the availability of clean water and good hygiene practices such as washing hands properly and the consumption of only properly cooked food is very helpful in controlling HEV virus spread[98].

In the case of immunocompromised patients, particularly in OT patients, intervention strategies should be considered in cases of acute or chronic HEV infection[99]. The first-line approach includes reducing of immunosuppressive medication because these drugs may influence viral replication and the course of liver disease. Oral ribavirin and pegylated-interferon have antiviral activity against HEV[59,100]. A twelve-week course of pegylated interferon, ribavirin or a combination of the two agents leads to viral clearance in about two-thirds of patients with chronic hepatitis E[100]; however, treatment failure may occur[59]. For patients with severe infection, ribavirin monotherapy should be considered to expedite viral clearance and recovery. Although ribavirin therapy is contraindicated in pregnancy owing to teratogenicity, the risks of untreated HEV to the mother and fetus are high and specific treatments are yet to be developed[100]. Given that specific therapies should be indicated for particular populations, the determination of the optimal dose, duration, and quantitative goals of ribavirin or pegylated-interferon treatment are still in progress[99].

CONCLUSION

In 2009, the WHO Expert Committee on Biological Standardization endorsed a proposal by the Paul-Ehrlich-Institut to prepare an International Standard for HEV RNA for use in NAT-based assays. This standard is currently available and should be implemented in all epidemiological studies with the aim of harmonizing methods globally[101].

To date, there are limited established guidelines and regulatory mechanisms for the study of highly dynamic diseases of public health impact, such as HEV, in Latin America. Careful scrutiny of the host distribution of the viruses that cause hepatitis E in Latin America, changes in the pathways of transmission, or the evolutionary dynamics of the genotypes of these circulating viruses, are required to support handling strategies of the disease, to define clinical treatments, and to prevent potential outbreaks. The common finding of this virus in epidemiological studies[102,103] represent a risk that must be properly handled.

Despite the widespread presence of HEV, this pathogen is not commonly considered from a global public health perspective. Limitations in medical and laboratory infrastructure and the absence of awareness of the disease caused by HEV may hinder surveillance studies and thus facilitate viral spread. There are now sensitive diagnostic assays and well-defined validation reagents to support the identification and preparedness to prevent HEV outbreaks[101]. A detailed guideline for following cases in endemic as well as in emergency situations needs to be developed to contain the virus (Figure 4). Active research on hepatitis E both in animals and humans has provided novel insights into HEV pathogenesis. More research is recommended to understand chronic and extra-hepatic infections to determine better treatment approaches.

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REVIEW

Genomic characterization of esophageal squamous cell carcinoma: Insights from next-generation sequencing

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Abstract

Two major types of cancer occur in the esophagus: squamous cell carcinoma, which is associated with chronic smoking and alcohol consumption, and adenocarcinoma, which typically arises in gastric reflux-associated Barrett's esophagus. Although there is increasing incidence of esophageal adenocarcinoma in Western counties, esophageal squamous cell carcinoma (ESCC) accounts for most esophageal malignancies in East Asia, including China and Japan. Technological advances allowing for massively parallel, high-throughput next-generation sequencing (NGS) of DNA have enabled comprehensive characterization of somatic mutations in large numbers of tumor samples. Recently, several studies were published in which whole exome or whole genome sequencing was performed in ESCC tumors and compared with matched normal DNA. Mutations were validated in several genes, including in *TP53*, *CDKN2A*, *FAT1*, *NOTCH1*, *PIK3CA*, *KMT2D* and *NFE2L2*, which had been previously implicated in ESCC. Several new recurrent alterations have also been identified in ESCC. Combining the clinicopathological characteristics of patients with information obtained from NGS studies may lead to the development of effective diagnostic and therapeutic approaches for ESCC. As this research becomes more prominent, it is important that gastroenterologist become familiar with the various NGS technologies and the results generated using these methods. In the present study, we describe recent research approaches using NGS in ESCC.

Key words: Esophageal squamous cell carcinoma; Next-generation sequencing; Somatic mutation; Driver mutation; Copy number variant

Core tip: Because targeted therapies have not been implemented in the treatment of esophageal squamous cell carcinoma (ESCC) to date, defining the genetic landscape of ESCC would facilitate the use of targeted therapies. Improvements in molecular profiling technologies have provided new insight into the basic molecular events during carcinogenesis as well as the mechanisms of anti-cancer drug resistance. Our invited review offers a current overview of the somatic genetic alterations in ESCC, emphasizing the recent results of large-scale sequencing efforts using next-generation sequencing technology.

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INTRODUCTION

Cancer of the esophagus is the eighth leading causes of cancer-related mortality worldwide[1]. It is one of the most deadly gastrointestinal tumors, with a 5-year survival rate of 20%-30% after curative surgery[2]. Two major types of cancer occur in the esophagus, including squamous cell carcinoma and adenocarcinoma, but their epidemiological features differ considerably. The incidence of esophageal cancer varies greatly by geographic location. Esophageal squamous cell carcinoma (ESCC) has a predilection for black and Asian populations and more than 70% of esophageal cancers worldwide are squamous cell carcinomas[3-5]. ESCC is considered an environmental malignancy attributable to chronic smoking and alcohol consumption[6]. In contrast, esophageal adenocarcinoma (EAC) predominantly affects white populations, which typically arises in a premalignant condition called Barrett's esophagus[7-10]. The changing epidemiology of esophageal cancer, with a dramatic increase in EAC and decrease in ESCC in Western countries indicates that differences exist between the two types of esophageal cancer[2].

Despite recent advances in imaging and surgical techniques, as well as the intensification of treatment with the increased use of chemoradiation, the survival rates for esophageal cancers have remained largely unchanged for several decades[6]. We have observed many patients with ESCC in whom local tumor recurrence or distant metastases occurred during an early disease stage and within a short period after surgery. Therefore, the molecular carcinogenesis and metastatic process of esophageal cancer must be clarified. Understanding tumor biology offers the potential for individualizing treatment and developing targeted therapies to increase cure rates and minimize morbidities. This review provides a current overview of the genomic and molecular characterization of ESCC, emphasizing recent results of large-scale sequencing efforts using next-generation sequencing (NGS) technology.

Since unique mutations have been observed in individual human cancer samples, the identification and characterization of molecular alterations underlying individual cancer patients is a critical step in the development of more effective, personalized therapies. For example, NGS technologies have revolutionized cancer genomics research by providing a comprehensive method for detecting somatic cancer genome alterations, such as point mutations, insertions, deletions, and copy number variations[11,12]. NGS has also revolutionized the field of genomics and improved our understanding of cancer biology. Advances have been achieved in the sequencing of tumor DNA; matched normal DNA was used to filter out germline variants to identify cancer-specific changes. High incidences of activating mutations in ESCCs amenable to drug targeting have also been identified. Investigators have also identified several critical genes and pathways important in the tumorigenesis of ESCC using this technology. This wealth of information undoubtedly improves our understanding of ESCC biology and provides clear targets for drug targeting to guide future personalized medicine.

TECHNICAL FEATURES OF NGS

NGS technologies have several advantages over classical Sanger sequencing, such as the ability to generate large quantities of DNA sequence information in a single run for detecting genetic mosaicism in depth[13]. However, routine usage of these technologies has several limitations, such as high cost, long processing time, and sample scalability. Three NGS platforms are now widely applied in cancer genome studies, including short-read technologies (< 400 bp) from Illumina (Genome analyzer/MiSeq/HiSeq/NextSeq; San Diego, CA, United States) and Thermo Fisher (SOLiD/Ion Torrent, Waltham, MA, United States) as well as a relative long-read technology (< 700 bp) from Roche (GS FLX, Basel, Switzerland). NGS platforms differ in performance metrics such as read length, accuracy, and output. The next next-generation (third-generation) sequencing system from Pacific Biosciences is also available (PacBio RS, Menlo Park, CA, United States), which can sequence a single molecule of DNA without polymerase chain reaction (PCR) amplification[14]. The average read length is 1500 bp, which is longer than that of any NGS technology, although the throughput of PacBio RS is lower than that of the second-generation sequencer. A brief summary of the technical features of these NGS platforms is shown in Table 1.

The NGS market is dominated by Illumina, which

Table 1 Commercial next-generation sequencing platforms for human genome sequencing

	454 GS FLX	GAIIx	MiSeq	HiSeq 2500	SOLiD 5500	Ion PGM (318 chip)	Ion Proton	PacBio RS
Reads per run	1 M	150 M	50 M	6 G	1.4 G	5.5 M	60 M	50 K
Read length (bp)	700	2 × 150	2 × 150	2 × 100	2 × 50	400	200	250-10000
Output per run	700 Mb	90 Gb	15 Gb	600 Gb	120 Gb	2 Gb	12 Gb	200 Mb
Run time	24 h	14 d	55 h	10 d	7 d	5 h	3 h	2 h
Cost/Mb[1]	$10.00	$0.15	$0.50	$0.05	$0.10	$1.00	$0.08	$2.00
Advantage	Long read length	Widly used	Widly used	High-throughput, widly used	High-throughput, accuracy	Fast, flexible chip	High-throughput, fast	Long read length, fast
Disadvantage	Long hand-on time, low output	Long run time	Long run time	Long run time	Long run time, short read length	High error rate (homopolymer)	High error rate (homopolymer)	High error rate

[1]Cost calculations are based on list price quotations obtained from the manufacturer. Mb: Megabase; Gb: Gigabase.

occupies the largest market share at 70% (www.marketsandmarkets.com). Illumina platforms are based on bridge amplification to clonally amplify the fragments, which are then sequenced using sequencing-by-synthesis chemistry[15]. Sequencing capabilities include both single-end sequencing and paired-end sequencing. The HiSeq 2000/2500 set the standard for high-throughput massively parallel sequencing. The original output was 200 Gb per run, which was improved to 600 Gb per run and can be finished in 10 d. The MiSeq was then released as a lower-throughput fast-turnaround instrument for use in smaller laboratories. Recently, Illumina developed the HiSeq X Ten Sequencing System, a very high-throughput and high-speed sequencing platform that enables sequencing for less than $1000 per genome at 30 × coverage[16].

After the human genome project, the first commercial NGS platform 454 pyrosequencer was developed by 454 Life Sciences Corp in 2005. The platform was purchased by Roche in 2007. Roche 454 platforms use emulsion PCR, and is based on pyrosequencing technology relying on the detection of pyrophosphate released during nucleotide incorporation[17]. The 454 GS-FLX system produces one million 700-bp sequences within 24 h. However, this platform has a significantly lower output compared to other NGS platforms. Additionally, the cost per base is also significantly higher compared with short-read technologies. The GS Junior is a benchtop version of this platform.

Similarly to Roche 454, the SOLiD sequencer relies on emulsion PCR and sequencing by ligation to small beads. Although the reads obtained from the SOLiD 5500 Genetic Analyzer system are only 50-75 bp in length, its system accuracy of 99.99% ranks first among all NGS platforms[18]. Recently, Ion Torrent sequencing technology based on semiconductor sequencing[19] has been released. Ion Torrent platforms use a high-density array of micro-machined wells, each containing a different DNA template. Beneath the wells lies an ion-sensitive layer, which is placed on a proprietary ion sensor to detect changes in pH resulting from incorporation of nucleotides in the new strand of DNA. The compact Ion Personal Genome Analyzer has three different chips, each designed for a specific purpose, including ranging from sequencing small genomes (314 chip, 550 K reads) and targeted gene sequencing (316 chip, 3 M reads) to chromatin immunoprecipitation-sequencing (ChIP-seq) (318 chip, 5.5 M reads). The desktop-type Ion Proton allows for larger chips with higher densities needed for the human exome and whole genome sequencing. The outstanding advantage of Ion Torrent is its speed: it takes 3-5 h from the start of sequencing until completion; however, this method has high error rate in homopolymer regions[20].

Applications of NGS for cancer genome research

NGS is increasingly used in many areas of cancer research and in the clinical setting. Depending on the purpose, NGS is applied in cancer genome studies, including whole genome, whole exome, targeted gene sequencing, RNA sequencing, and ChIP-Seq[11,12,21-23]. For variant identification by resequencing target regions, whole genomes, or whole exons, it is key to sequence both the tumor and non-malignant tissues of an individual. There are 3-5 million inherited sequence variants per human genome. Consequently, most sequence variants identified in a cancer genome are inherited polymorphisms and are not somatic mutations[24]. Thus, comparing a tumor genome to its paired normal genome is required to efficiently identify somatic sequence variants (Figure 1). In addition to CGH and SNP arrays, NGS techniques can be used to detect copy number variations[12,25]. Targeted sequencing is a variation of re-sequencing where only a small subset of the genome is sequenced, such as a set of genes or particular sequences under interest. Although this approach will not detect most structural

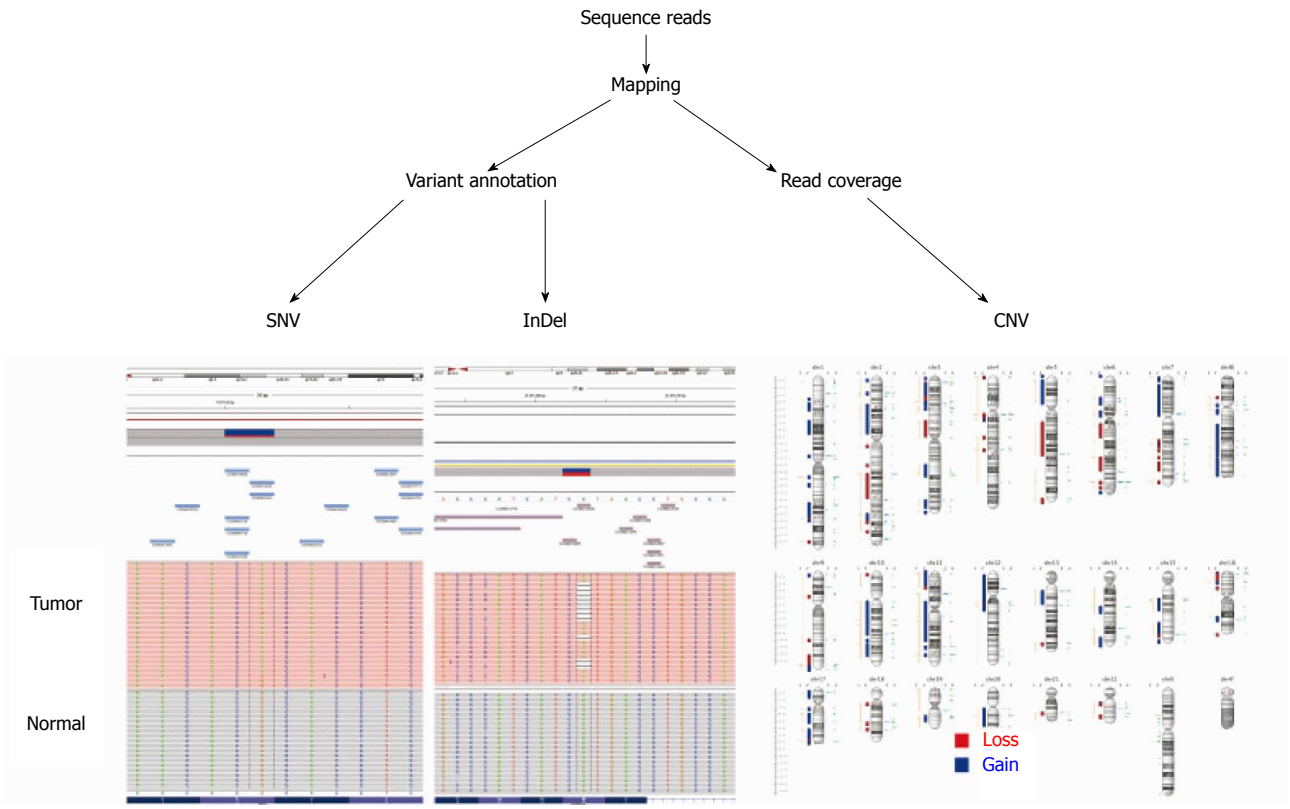

Figure 1 Cancer genome research using next-generation sequencing. Sequence reads are quality checked and then mapped to the reference genome. Somatic DNA alterations are detected using statistical approaches in tumor and normal samples from the available software or an integrated workflow such as the GATK pipeline (Broad Institute). SNV: Single nucleotide variant; InDel: Insertion and deletion; CNV: Copy number variation.

Table 2 Next-generation sequencing studies of esophageal squamous cell carcinoma to date

Study	Method	Sample number	Number of non-silent mutations/tumor	Additional analyses	Platform
Agrawal *et al*[31]	WES	12 WES	83	-	GA IIx
Song *et al*[34]	WGS and WES	17 WGS, 71 WES	61	123 CGH	HiSeq 2000
Lin *et al*[33]	WES and TS	20 WES, 119 TS	59	4 RNA-seq, 59 CGH, 125 SNP-array	HiSeq 2000
Gao *et al*[32]	WES	113 WES	82	-	HiSeq 2000
Zhang *et al*[35]	WGS and WES	14 WGS, 90 WES	104	-	HiSeq 2000

WGS: Whole-genome sequencing; WES: Whole-exome sequencing; TS: Targeted sequencing; CGH: Comparative genomic hybridization; RNA-seq: RNA sequence; SNP: Single nucleotide polymorphism.

variants, such as chromosomal translocations, targeted gene sequencing represents a cost- and resource-efficient approach for identifying somatic mutations in cancer genomes[26,27].

GENETIC ALTERATIONS DRIVING ESCC

Targeted therapies have been successfully used for the treatment of in certain human solid tumors, including lung adenocarcinoma, colorectal cancer, stomach cancer, breast cancer and renal cell carcinoma as well as hematologic malignancies, but have not been implemented in the treatment of ESCC[28-30]. Therefore, defining the genetic landscape of ESCCs would facilitate the use of targeted therapies. Agrawal and colleagues published the first exome-sequencing study of esophageal cancer, sequencing 12 ESCCs and 11 EACs as well as matched non-neoplastic tissues from subjects in the United States[31], and a handful of NGS studies in ESCC have been published over the last four years (summarized in Table 2)[31-35]. Genetic aberrations identified within these studies, including gene mutation, gene rearrangement, and gene amplification/deletion, increased the understanding of constitutive activation of oncogenes, or loss of function of tumor suppressors. These comprehensive studies have demonstrated recurrent mutations in several genes in ESCC, most notably *TP53*, *NOTCH1*, PIK3CA and *FAT1* as well as copy-number alterations in *CCND1* and *CDKN2A* (Table 3). Figure 2 shows a Venn diagram of the most significantly mutated genes identified in the three whole genome and whole exome

Table 3 Frequently altered genes in esophageal squamous cell carcinoma

Gene symbol	*TP53*	*NOTCH1*	*PIK3CA*	*CDKN2A*	*CCND1*	*FAT1*
Chromosomal location	17p13.1	9q34.3	3q26.3	9p21.3	11q13	4q35.2
Alteration frequency (%)						
Agrawal *et al*[31]	92 (M)	33 (M)	0 (M)	8 (M)	NA	8 (M)
Song *et al*[34]	83 (M) 1 (L)	9 (M)	5 (M) 41 (G)	5 (M) 44 (L)	46 (G)	5 (M)
Lin *et al*[33]	60 (M)	8 (M)	7 (M) 10 (G)	3 (M) 33 (L)	46 (G)	12 (M)
Gao *et al*[32]	93 (M)	14 (M)	9 (M) 2 (G)	8 (M) 12 (L)	33 (G)	11 (M)
Zhang *et al*[35]	88 (M)	21 (M)	17 (M)	8 (M) 64 (L)	64 (G)	15 (M)

M: Nonsynonymous mutation; L: Copy number loss; G: Copy number gain; NA: Not applicable.

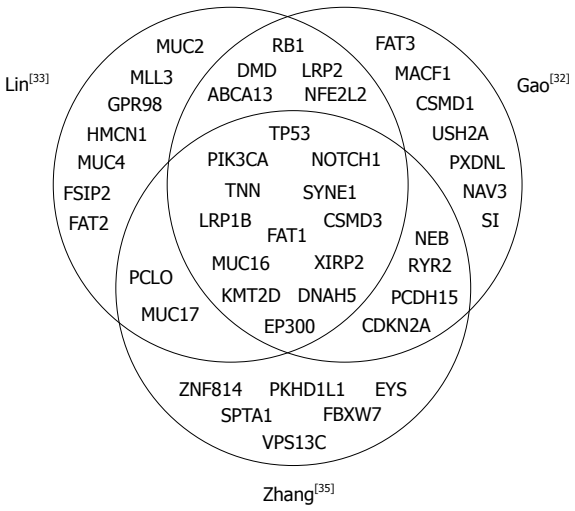

Figure 2 Comparison of most significantly mutated genes identified in the three whole genome and whole exon sequencing studies for esophageal squamous cell carcinomas. Top significantly mutated genes (25-27 genes per study) were obtained from three recent next-generation sequencing studies of large cohorts. Only nonsynonymous mutations were considered.

sequencing studies of relatively large cohorts for ESCCs. The Broad Institute (Cambridge, MA, United States) project[36] used this method to examine 149 EAC tumors, and confirmed recurrent driver mutations in *TP53*, *CDKN2A*, *SMAD4*, *ARID1A*, and *PIK3CA*. Previously unidentified mutations in *SPG20*, *TLR4*, *ELMO1*, and *DOCK2* were also found, and a possible role for the RAC1 GTPase pathway was identified. The genomic landscape of EAC differed from that of ESCC, highlighting the different therapeutic strategies needed to treat esophageal cancers. In this review, we highlight the current knowledge regarding molecular targets, clinical trials of targeted agents, and druggable aberrations in ESCCs.

p53 family

TP53 is one of the most thoroughly studied tumor suppressor genes in human cancer. Genetic mutations in *TP53* are present in more than 50% of human cancers, leading to dysregulation of its downstream targets[37,38]. NGS studies have confirmed that *TP53* is the most commonly mutated gene in ESCC. The first whole exome sequencing study found that ESCCs contained an average of 83 mutations per tumor, and that the most frequent mutations in ESCC occurred in *TP53* (92% of the 12 cases sequenced), *NOTCH1* (33%), *NOTCH3* (25%), and *FBXW7* (17%)[31]. The p53 protein is activated by a variety of cell stresses, such as DNA damage, oncogene activation, spindle damage, and hypoxia. Activated p53 transactivates a number of target genes, many of which are involved in DNA repair, cell cycle arrest, and apoptosis[39-41]. *TP53* alterations have been identified as early events in the carcinogenesis of ESCC and have been associated with disease progression and a poor outcome[42-44]. Therapies targeting TP53 loss of function are currently being examined in clinical trials, and several studies suggest that patients harboring *TP53* alterations will respond better to angiogenesis inhibitors[45]. The efficacy of intra-tumor injection of p53 adenovirus (Advexin, Introgen Therapeutics Inc., Austin, TX, United States) has been confirmed in Japanese ESCC patients[46]. Additionally, a TP53 adenoviral-based treatment (Gendicine, Shenshen Sibiono Genetech, Shenzhen, China) for patients with squamous cell carcinoma of the head and neck has recently been approved for use in China[47]. Two other p53 family members, p63 and p73, also induce cell cycle arrest and apoptosis and play an important role in development and differentiation[48]. Dominant negative forms lacking the N-terminal transactivation domain (DNp63 and DNp73) are overexpressed in some types of cancers[49]. In esophageal cancer, DNp63 is overexpressed in ESCC but not in EAC, making p63 a useful marker of squamous cell cancer[50,51]. Additionally, at least 30% of head and neck squamous cell carcinomas harbor mutations in genes regulating squamous differentiation, including *p63*[52].

Notch signaling

NOTCH1 is the second most commonly mutated gene in ESCC, with a mutation rate of 8%-33%[31-35]. The

Notch signaling pathway is thought to play important roles in regulating normal cell differentiation in a context-dependent manner[53]. The Notch pathway has also been implicated in human carcinogenesis as both an oncogene and a tumor suppressor[54]. The oncogenic activity of this pathway has been observed in a number of hematopoietic cancers[55]. When we characterized the distribution of *NOTCH1* somatic mutations obtained from the two studies[32,35], most *NOTCH1* mutations observed in ESCC affect the epidermal growth factor (EGF)-like ligand-binding domain (56%, 30 of 54) and are thought to lead to a loss of function. Inactivating mutations in these regions of the gene have also been observed in cutaneous, lung, head, and neck squamous cell carcinomas[56-58]. Thus, the idea that the same gene can function in completely opposite manners in different cell types is important for understanding cell signaling pathways. In addition to *NOTCH1*, mutations in the *NOTCH2* and *NOTCH3* genes were detected in ESCC[31,32]. Interestingly, Agrawal *et al*[31] identified inactivating mutations of *NOTCH1* in 21% of ESCC but not in EAC, suggesting tumor-suppressive roles of Notch signaling in squamous cell carcinomas. Notch pathway disruption also results from *FBXW7* mutations, which were identified in 5%-17% of ESCC specimens, because FBXW7 forms part of the ubiquitin ligase complex that mediates NOTCH1 degradation[59].

RTK-MAPK-PI3K pathway

KRAS is one of the most frequently mutated oncogenes in human cancer[60]. In ESCC, *KRAS* mutations are generally rare[61], although the incidence of *KRAS* mutations in Chinese patients with ESCC was relatively high, with a mutation rate of 12%[62]. Receptor tyrosine kinases (RTKs) of the EGFR family are involved in development and progression of epithelial tumors and thus represent therapeutic targets for inhibition by tyrosine kinase inhibitors or humanized monoclonal antibodies[63-65]. Upstream RTKs, EGFR, ERBB2, ERBB4, and MET, as well as G-protein-coupled receptors activate phosphoinositide-3-kinase (PI3K) after binding of growth factor ligands[63]. The PI3K pathway plays a key role in regulating multiple cellular events, including cell growth, proliferation, cell cycle progression, and survival[66,67]. *PIK3CA* is the second most commonly mutated gene, occurring frequently (< 20%) in most cancer types[60]. Overexpression of EGFR has been described in ESCC; most ESCC tumors show increased activity in the absence of somatic mutations[68-70]. In addition to *EGFR* amplification, this pathways displayed genetic alterations in 78.6% of cases, including *FGFR1*, *ERBB2*, *RAF1*, *AKT1*, *SOS1*, *SOS2*, and *PIK3CA* mutations and amplifications[33]. Moreover, EGFR transactivation *via* ectodomain shedding of EGFR ligands plays a role in inflammation as well as tumor growth and metastasis[71,72]. A recent report demonstrated that targeting the sheddase activity of ADAM17, which is responsible for the release of multiple EGFR ligands, decreased head and neck squamous cell carcinoma cell viability and motility through blocking of the EGFR pathway[73]. Since RTKs are well-characterized druggable proteins, targeting components in this pathway may represent valuable investigational avenues for clinical trials in patients with ESCCs. Of interest, *KRAS*, a frequently aberrant gene in non-squamous tumors that leads to resistance to PI3K pathway inhibitors, was found to be aberrant significantly less frequent in ESCCs. Recent clinical studies have demonstrated that anti-EGFR monoclonal antibody (cetuximab) in combination with irradiation yielded encouraging survival and local control in ESCC patients[74].

Cell cycle regulation

The cell cycle regulation pathway is one of the most perturbed pathways in ESCC. Mutations have been observed in the cell-cycle regulatory pathway genes *TP53* (88%), *CDKN2A* (8%), and *RB1* (2%)[35]. In addition, ESCC tumors show amplification of *CCND1*, which encodes for cyclin D1, and deletion of *CDKN2A/B*, which encodes for p16 and p14. Cyclin D1 is responsible for inducing the G1/S phase transition and is located at 11q13[31-35]. Gains in the chromosomal region 11q13 are some of the most prominent genetic alterations in squamous cell carcinomas and are associated with poor prognosis and metastasis[28,75,76]. Other G1/S transition control molecules, *CDK4*, *CDK6*, *E2F1*, and *MDM2*, are also amplified in ESCC. The p16 tumor suppressor can inhibit the formation of the CDK4/6 and cyclin D1 complex and plays a role in the oncogene-induced senescence of cells. Gain of *CCND1* and/or loss of *CDK2NA* events occurred in over 70% of ESCC samples[33-35]. Flavopiridol, the first cyclin-dependent kinase inhibitor examined in human clinical trials, was reported to be a targeting drug for ESCC and head and neck squamous cell carcinoma patients[77]. *NFE2L2*, a frequently mutated gene in ESCC, encodes a sequence-specific transcriptional factor that upregulates genes associated with oxidative stress. Activating missense mutations in the *NFE2L2* gene result in accumulation of the NFE2L2 protein and promote aberrant activation of downstream genes that confer resistance to oxidative stress and induce metabolic transformation in cancer cells[78].

Other signaling pathways

Altered genes in the Wnt pathway were also frequently found in ESCC, including mutations in CTNNB1 and SFRP4, and mutations and amplifications of AXIN inhibitors, DAAM2, DVL3, LRP5 and LRP6[34]. In addition, loss of FAT1, by either somatic mutation or deletions, promotes tumorigenesis through activation of Wnt signaling[79].

Dysregulation of proteins involved in chromatin regulation can affect the genome-wide control of

gene expression and play key roles in DNA repair and genome maintenance. Mutations in a number of genes involved in histone modifications have been identified in many cancer types[60]. Inactivating missense mutations in several chromatin-remodeling genes, including *EP300*, *CREBBP*, and *BAP1*, in ESCC samples. Moreover, truncating mutations were observed in the chromatin-remodeling genes *KMT2D*, *KMT2C*, and *KDM6A*[35]. Approximately 30% of ESCC tumors contained at least one chromatin remodeling gene alteration.

Recently, inactivating mutations in the Hippo pathway regulator (*AJUBA*, *FAT1*, *FAT2*, *FAT3*, *and FAT4*) were observed in ESCC[32]. Hippo signaling cross-talks with commonly mutated cancer genes such as *KRAS, PIK3CA, CTNNB1*, or *FBXW7*[80,81].

CONCLUSION

The identification and characterization of molecular alterations in individual cancer patients is a critical step towards the development of more effective personalized therapies. NGS technologies have revolutionized cancer genomics research by providing a comprehensive method of detecting somatic DNA modifications. ESCC is the major histological type of esophageal cancer in East Asian countries and is one of the most aggressive malignant tumors. Recent studies using NGS have revealed that ESCC is characterized by specific somatic DNA modifications such as exonic mutations, copy-number alterations, and genomic rearrangements. The most common mutation in ESCC is TP53. Pathway assessment has shown that somatic aberrations within ESCC genomes are mainly involved in several important pathways, including cell cycle regulation and the Notch, RTK-MAPK-PI3K, and Wnt pathways. We expect that many new discoveries will increase our understanding of the molecular mechanisms of ESCC for targeted therapies.

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REVIEW

Role of Tim-3 in hepatitis B virus infection: An overview

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Abstract

Hepatitis B virus (HBV) infection has received increasing public attention. HBV is the prototypical member of hepadnaviruses, which naturally infect only humans and great apes and induce the acute and persistent chronic infection of hepatocytes. A large body of evidence has demonstrated that dysfunction of the host anti-viral immune response is responsible for persistent HBV replication, unresolved inflammation and disease progression. Many regulatory factors are involved in immune dysfunction. Among these, T cell immunoglobulin domain and mucin domain-3 (Tim-3), one of the immune checkpoint proteins, has attracted increasing attention due to its critical role in regulating both adaptive and innate immune cells. In chronic HBV infection, Tim-3 expression is elevated in many types of immune cells, such as T helper cells, cytotoxic T lymphocytes, dendritic cells, macrophages and natural killer cells. Tim-3 over-expression is often accompanied by impaired function of the above-mentioned immunocytes, and Tim-3 inhibition can at least partially rescue impaired immune function and thus promote viral clearance. A better understanding of the regulatory role of Tim-3 in host immunity during HBV infection will shed new light on the mechanisms of HBV-related liver disease and suggest new therapeutic methods for intervention.

Key words: Tim-3; Hepatitis B virus; Inflammation; Immunity; Liver disease

Core tip: Here, we discuss the current knowledge of the interaction between hepatitis B virus (HBV) and host immunity, addressing the important role of T cell immunoglobulin domain and mucin domain-3 (Tim-3) in HBV infection. Tim-3 expression on both adaptive and innate immune cells is elevated in HBV infection. Increasing Tim-3 expression inhibits, and blocking Tim-3 expression rescues, the anti-viral immune

response, indicating that Tim-3 is a potential target for controlling HBV infection. Finally, we describe remaining unsolved problems in this field and analyze the potential of Tim-3 as a novel drug target in the treatment of HBV-related liver diseases.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a well-known and increasingly severe public health problem worldwide. According to epidemiological data, the number of people with resolved or present HBV infection has reached an alarming 2 billion[1,2]. For most patients with chronic HBV infection, the present drug treatment is incapable of thoroughly eliminating the virus, owing to HBV DNA integration into the host genome and the formation of covalently closed circular DNA structures[3]. Furthermore, many patients are threatened by a lifetime 15%-40% risk of developing HBV-related cirrhosis, liver failure and hepatocellular carcinoma (HCC)[1,2]. The HBV genome contains 3200 bp and forms a relaxed circular, partially double-stranded structure[4]. The HBV genome contains 4 compact overlapping open reading frames encoding different viral proteins: preS/S, preCore/Core, polymerase (pol) and X. Because of multiple alternative start codons, surface proteins exist in 3 forms, termed small, medium and large surface proteins, which are needed for virion assembly. The core protein forms the viral nucleocapsid and has a secreted counterpart termed e antigen. The polymerase is a multi-functional enzyme that serves as a DNA-dependent DNA polymerase, reverse transcriptase and RNase H. *X* is the smallest gene of HBV; this gene is composed of 452 nucleotides and encodes a 17-kDa protein[5]. A large body of evidence has demonstrated that HBV can cooperate with other etiological factors and then trigger tumorigenesis and the development of HCC. Thus, suppression of HBV DNA replication and the clearance of viral products are the main goals of HBV treatment.

Considerable evidence has shown that host immunity is responsible for the control of HBV infection and is the primary determinant of HBV disease progression. Impaired function of adaptive immunocytes, particularly HBV-specific $CD8^+$ T cells, is considered to be the primary cause of widespread viral infection. HBV tends to stimulate an immunosuppressive environment that is beneficial for its survival. For example, HBV infection increases the number of regulatory T cells (Tregs), which repress effector T cell activity[6]. However, impairments in the adaptive immune response cannot explain all events that occur during HBV infection, because various components of the innate immune system also participate in disease progression. Indeed, the activation of dendritic cells (DCs), natural killer cells (NKs) and macrophages during acute infection leads to a bona fide clinical outcome, whereas persistent HBV infection at least partly results from dysregulation of the innate immune response at early stages of infection[7]. Therefore, studying the interaction between HBV and host immunity and uncovering the reason why the immune response is dysregulated in HBV infection are critical.

Innate and adaptive immunocyte activation is regulated by a set of inhibitory surface receptor-ligand pairs, or immune checkpoints. Among these pairs, T cell immunoglobulin domain and mucin domain-3 (Tim-3) and its matched ligand are currently attracting increasing attention because of their demonstrated potential as a target for immunotherapy for infectious diseases and cancers. Although Tim-3 was first identified as a surface molecule specifically expressed on $CD4^+$ T helper 1 (Th1) and $CD8^+$ type 1 (Tc1) cells[8], further studies have revealed that Tim-3 is also expressed on many other cell types undergoing dynamic changes during infection. In the resting state, Tim-3 is expressed on only a very small percentage of $CD4^+$ or $CD8^+$ T cells, and its over-expression may indicate T cell exhaustion and represent a pathological immune state[9]. However, innate immune cells including monocytes, macrophages and DCs show constitutive and high-level Tim-3 expression that can be further elevated in some diseases. Tim-3 is the prototypical member of the Tim family, which includes 8 members (Tim-1- Tim-8) in mice and 3 members in humans (Tim-1, -3, -4). Tim family members share a similar molecular structure consisting of 4 parts: an N-terminal IgV domain, a mucin domain, a transmembrane domain and a cytoplasmic tail[9]. Galectin-9 (Gal-9), a widely expressed S-type lectin, was the first identified ligand for Tim-3. The interaction of Tim-3 with Gal-9 leads to apoptosis of Th1 cells and inhibition of Th1 and Tc1 cell-mediated immunity[10]. Emerging evidence has shown that additional Tim-3 ligands exist, including phosphatidylserine, carbohydrate moieties and the alarmin high-mobility group box 1[11]. Carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1), another membrane molecule that inhibits T cell activation, is a newly identified ligand for Tim-3. Binding of Tim-3 and CEACAM1 appears to be necessary for the T cell inhibiting function of Tim-3, and this interaction has a crucial role in regulating anti-tumour immunity[12]. Thus, the interactions of Tim-3 with its ligands play important roles in different immune-related diseases by regulating both innate and adaptive immunity.

Although Tim-3 has gained public attention as an inhibitory immune regulator, its role in regulating the host immune response is complicated and remains controversial in several fields, particularly in infectious diseases.

This review will briefly describe the function of Tim-3 in regulating immunity, which has become a hot topic of research in recent years. In addition, this review will discuss in detail the important role of Tim-3 in HBV infection, because studies in this field are currently lacking.

TIM-3 AND THE ADAPTIVE IMMUNE RESPONSE IN HBV INFECTION

Tim-3 and effector T cells

T cell exhaustion, which is characterized as low proliferative ability, decreased cytokine production and suppressed cytotoxicity, often occurs in individuals with chronic viral infections including HBV, hepatitis C virus (HCV) and human immunodeficiency virus (HIV). T cell exhaustion has been detected in both animal models and clinical patients. In particular, dysfunction of antigen-specific $CD8^+$ T cells is believed to be one of the most important reasons why viruses such as HBV, HCV and HIV escape from the anti-viral immune response. One of the key characteristics of exhausted T cells is the combined over-expression of several inhibitory surface markers, such as programmed death 1 receptor(PD-1), lymphocyte-activation gene 3 (LAG-3) and Tim-3[13,14]. Although PD-1 has been extensively studied, Tim-3 has attracted increasing attention in recent years. However, the role of Tim-3 in infectious diseases remains unclear.

The identification of Tim-3 as a negative regulator of the anti-viral adaptive immune response was first reported in chronic HIV infection. The numbers of $Tim\text{-}3^+CD8^+$ T and $Tim\text{-}3^+CD4^+$ T cells are increased in patients infected with HIV[15,16], and compared with $Tim\text{-}3^-$ cells, $Tim\text{-}3^+CD4^+$ and $Tim\text{-}3^+CD8^+$ T cells show impaired functions. Furthermore, blocking Tim-3 with a neutralizing antibody rescues the anti-viral immune response to a certain extent[15].

In 2009, our laboratory was the first to demonstrate the crucial role of Tim-3 in inhibiting hepatic $CD8^+$ T cells in HBV infection. In a mouse model with hydrodynamic injection of HBV-bearing plasmids, augmented Tim-3 expression was detected on hepatic $CD8^+$ T cells which displayed decreased interferon (IFN)-γ production. Furthermore, Tim-3 silencing enhanced IFN-γ production and even indirectly affected HBV neutralizing antibody production, suggesting the potential role of Tim-3 in the host antiviral immune response against HBV infection[17].

Two years later, Wu *et al*[18] studied the relationship between Tim-3 expression on peripheral T cell subsets and disease progression in patients with chronic hepatitis B (CHB). They found that Tim-3 expression is increased on $CD4^+$ and $CD8^+$ T cells, and its expression is associated with the severity of CHB. Tim-3 expression may also indicate the severity of liver injury because its expression has been found to be markedly and positively correlated with alanine aminotransferase (ALT), aspartate aminotransferase (AST), the international normalized ratio (INR) and total bilirubin (TB). After control of CHB infection, Tim-3 expression decreases. Moreover, the percentage of $Tim\text{-}3^+$ T cells is negatively correlated with plasma IFN-γ and T-bet mRNA levels, indicating that high levels of Tim-3 expression inhibit T cell activity[18].

In 2012, the same group investigated the immunocompetence of $Tim\text{-}3^+CD8^+$ and $Tim\text{-}3^-CD8^+$ T cells and the effects of Tim-3 expression on lymphocyte proliferation and cytokine secretary capacity. Compared with $Tim\text{-}3^-CD8^+$ cells, $Tim\text{-}3^+CD8^+$ T cells show a lower capacity to proliferate and produce cytokines upon antigen challenge. Interference of the Tim-3 pathway by either anti-Tim-3 antibodies or Tim-3 short hairpin RNAs rescues $CD8^+$ T cell activity, improving their proliferation and enhancing their cytokine secretary capacity, which suggests the potential of Tim-3 to become a new drug target for controlling HBV infection[19].

Similar results have been observed in HCV infection. During chronic HCV infection, Tim-3 expression on $CD4^+$ and $CD8^+$ T cells is elevated, and these $Tim\text{-}3^+$ T cells exhibit a $CD127^{low}CD57^{high}$ and $CD45RA^-CCR^{high}$ phenotypes, indicating the impaired function of these effector cells. Accordingly, blockade of Tim-3 expression enhances cell proliferation and promotes cytokine production[20]. The level of Tim-3 expression on $CD4^+CD25^+$ T cells is negatively correlated with the proliferative capacity of effector T cells, and blocking Tim-3 results in the rapid expansion of these cells[21]. This finding has been further confirmed in studies with HCV vaccination. Upon stimulation with live-attenuated HCV vaccine, effector T cells isolated from patients infected with HCV display a diminished anti-viral response compared to those isolated from healthy individuals. Furthermore, Tim-3 blockade substantially rescues the impaired function of $Tim\text{-}3^+$ T cells, indicating that Tim-3 is responsible for the peripheral tolerance during persistent HCV infection. Of note, the recovery of T cell function after Tim-3 blockade may at least partially result from an enhanced antigen presentation ability of DCs because researchers have also found that Tim-3 may inhibit DC maturation during chronic HCV infection[22].

The co-expression of Tim-3 and other inhibitory regulators such as PD-1 in HBV and HCV infections is also important. Studies have shown that $PD\text{-}1^+$ $Tim\text{-}3^+$ T cells are abundant among the central memory T cell subsets, particularly in the liver, during chronic HCV infection. Moreover, compared to HCV mono-infection, the percentage of $PD\text{-}1^+Tim\text{-}3^+$ T cells is much higher in patients co-infected with HIV and HCV. Co-

expression of PD-1 and Tim-3 on HCV-specific T cells may also reflect liver disease progression. Although the function of PD-1 and Tim-3 co-expression remains unclear, researchers have hypothesized that co-expression of various inhibitors may increase the risk of persistent and refractory virus infection[23].

Although Tim-3 has been well recognized as a marker of T cell exhaustion and a negative regulator of adaptive immunity in chronic virus infection, its roles in acute viral infection and bacterial infection are different. Elevated Tim-3 expression is observed on T cells in the early stage of acute hepatitis B (AHB); however, this elevation is transient and quickly reversed at the convalescence stage. Moreover, unlike CHB, the level of Tim-3 expression is not correlated with either different stages of hepatic injury or serum IFN-γ levels in patients with AHB[19]. In active TB infection, Tim-3 is up-regulated on both $CD4^+$ and $CD8^+$ T cells; however, in contrast to chronic virus infection, Tim-3^+ effector T cells show more active anti-TB responses. One possible explanation for this finding is that Tim-3 is not only a marker of T cell exhaustion but also a marker of T cell differentiation, because CD127 is also expressed on these cells, indicating the complicated roles of Tim-3 in different microenvironments[24]. Similar results have been observed in mice infected with *Listeria monocytogene*s. Tim-3 expression is induced in cytotoxic T cells in infected wild-type mice, and the host exhibits a much stronger immune response compared to that in Tim-3-knockout mice[25]. Because other members in the Tim family may substitute for Tim-3 in Tim-3-deficient mice, the hypothesis that Tim-3 may play an important positive role in the adaptive immune response should be considered.

Tim-3 and Treg/Th17 cells

Tregs, defined as $CD4^+CD25^+FOXP3^+$ regulatory T cells, can repress the functions of other immune cells *via* cell-to-cell contact and secretion of immuno-suppressive cytokines such as transforming growth factor (TGF)-β and interleukin (IL)-10[26]. Th17 cells are a new subtype of $CD4^+$ T cells that secrete the cytokine IL-17[27]. Accumulating data support the negative roles of these 2 cell types in chronic viral infections including HBV. Indeed, the proportion and absolute numbers of both cell types are increased in both peripheral blood mononuclear cells and liver tissues in patients with CHB[28-32]. The number of circulating Th17 cells is also positively associated with the levels of liver injury markers[33]. Moreover, the differentiation pathways of Th17 cells and Tregs are controlled by similar cytokines, and the Treg/Th17 ratio is strongly associated with HBV load. Furthermore, imbalance of the Treg/Th17 ratio is involved in HBV-related diseases and may become a novel drug target in the future[34,35]. HBV has also been reported to enhance the function of Tregs. In particular, co-culture of T cells with HepG2.2.15 cells, a hepatoma cell line stably integrated with the HBV genome, promotes Treg development and induces the expression of Treg-related genes[36].

The Tim-3/Gal-9 interaction is known to be involved in Treg function. Accordingly, blocking the Tim-3-Gal-9 pathway results in an obvious decrease in the suppressive activity of Tregs *in vitro*[37,38]. In mice, Tim-3 is constitutively expressed on natural Treg cells. In contrast, Tim-3 is not expressed on human Treg cells *ex vivo* but is up-regulated after activation. Tim-3^+ Treg cells also display increased expression of other inhibitory receptors including LAG-3, cytotoxic T-lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor and PD-1[37]. In addition, Tim-3 has been identified as a marker of Tregs in tumors[39].

Tim-3 is over-expressed in Tregs in patients chronically infected with HCV. Tim-3^+ Tregs tend to resist apoptotic signals and show a higher capacity to proliferate, leading to Treg accumulation. Moreover, Ji *et al*[40] have reported that HCV infection leads to elevated Gal-9 and TGF-β production in hepatocytes. Co-culture of HCV-infected hepatocytes and $CD4^+$ T cells induces increased Tim-3 expression on $CD4^+$ T cells, and the Tim-3/Gal-9 interaction enhances TGF-β/IL-10 production by $CD4^+$ T cells, which accelerates the differentiation of $CD4^+$ T cells into Tregs[40]. However, the regulatory effects of Tim-3 on Tregs in HBV infection remain to be clarified.

Tim-3 has also been reported to be involved in regulating Th17 cells. Tim-3 appears to suppress the activation and cytokine secretion of Th17 cells, and Tim-3 expression is impaired in many auto-immune diseases such as Guillain-Barré syndrome and psoriasis[41,42]. However, few studies have focused on the expression pattern of Tim-3 on Th17 cells during persistent HBV or HCV infection, which highlights significant gaps in this research field.

In summary, during chronic HBV infection, Tim-3 is induced in adaptive immune cells and represses host anti-viral immunity. Furthermore, blocking Tim-3 seems to be beneficial for controlling viral activity and may become a future therapeutic approach for treating CHB (Figure 1).

TIM-3 AND THE INNATE IMMUNE RESPONSE IN HBV INFECTION

Because of the key role of adaptive immunity, the role of innate immunity in HBV infection has largely been ignored in previous studies. However, increased understanding of pathogen-associated molecular patterns and signaling pathways that control the activation of innate immunity has highlighted the importance of the innate immune system in HBV-related diseases[7]. A large body of evidence has proved that Tim-3 regulates innate immune response. Unlike adaptive T cells, innate immune cells such as

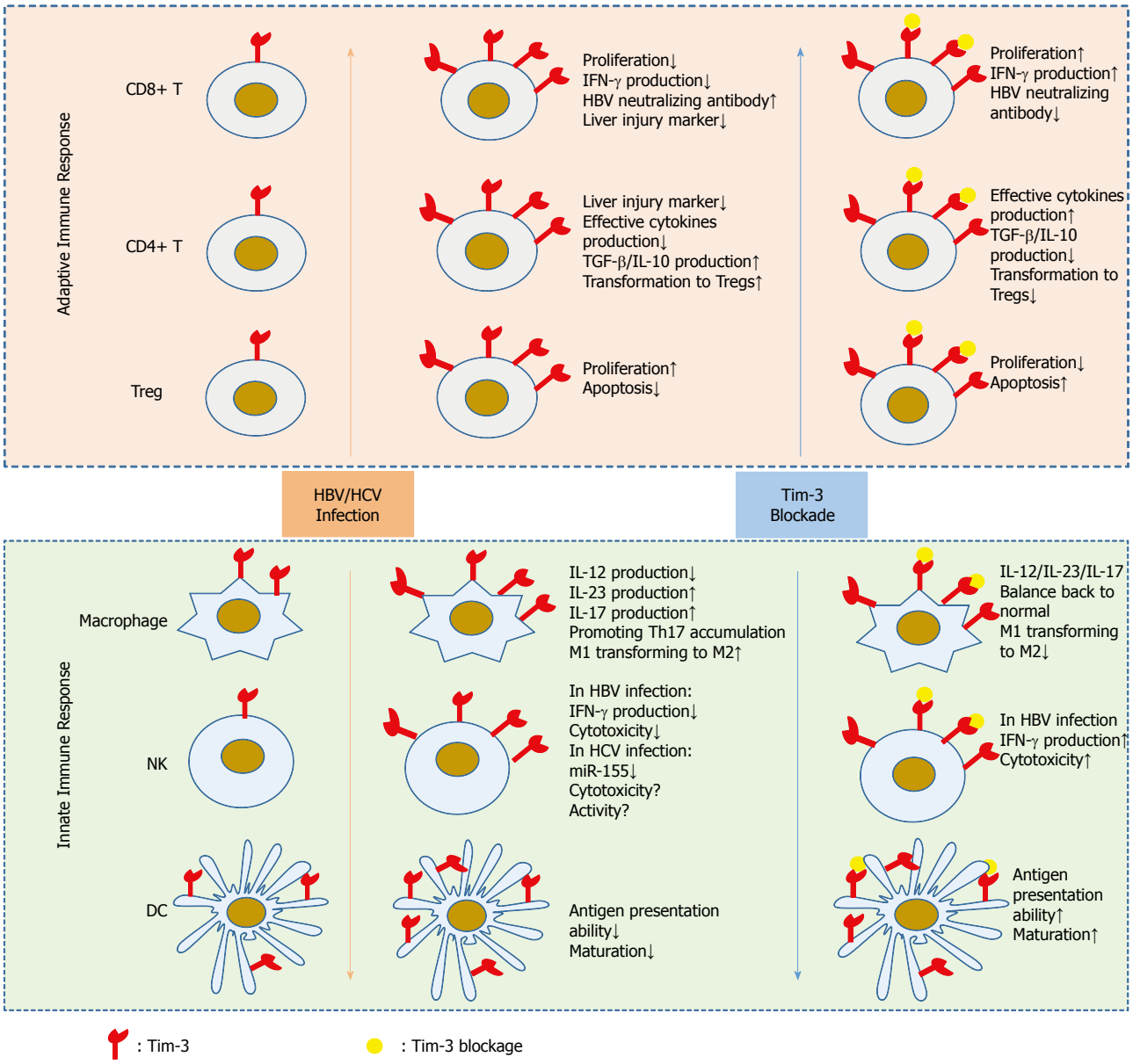

Figure 1 Role of Tim-3 in hepatitis B virus infection. In the resting state, Tim-3 expression on most adaptive and innate immune cells is low, except for dendritic cells and macrophages, which show high and stable Tim-3 expression. However, chronic HBV/HCV infection induces or further enhances Tim-3 expression on those cells and inhibits their anti-viral immune response. Blocking Tim-3 helps rescue the impaired function of these cells and promotes virus clearance, indicating its potential as a drug target for the treatment of virus-related liver diseases. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

monocytes/macrophages, NKs and NK T cells (NKTs) constitutively express Tim-3, which can be further elevated in some diseases including chronic viral infection. Interference of Tim-3 pathway changes the function of innate immune cells[43,44].

Tim-3 and monocytes/macrophages

Monocytes and macrophages are important components of the innate immune system. Upon stimulation with inflammatory signals, monocytes rapidly infiltrate sites of infection and then differentiate into macrophages that kill pathogens. In HBV infection, macrophages are crucial in modulating chronic liver injury and HBV clearance. Macrophages can be classified into 2 types: M1 and M2. M1 cells contribute to HBV clearance, whereas M2 cells impair the host immune response, promote HBV infection and accelerate tumorigenesis[45,46]. In addition, HBV tends to promote M2 polarization[6,7]. Several studies have reported that pathogens can induce Tim-3 overexpression in macrophages and monocytes, which may regulate the activation and cytokine production of these cells[47]. Moreover, lipopolysaccharide, a Toll-like receptor (TLR) ligand, can repress Tim-3 expression on macrophages and at least partially rescue their function, suggesting that TLRs and their downstream pathways may be involved in the regulation of Tim-3 expression[48]. Of note, the role of Tim-3 in natural immunity is much more complex than its role in adaptive immunity. Recently, our laboratory has found

that Tim-3 regulate the polarization of macrophages and accelerate the transformation of M1 macrophages into M2 macrophages, which then suppress the inflammatory response in HCC[44]. Together, these data indicate the complicated role of Tim-3 in regulating innate immune cells.

Tim-3 expression has been reported to be strongly elevated on monocytes in patients with CHB and further elevated in those patients with acute-on-chronic liver failure (ACLF). Tim-3 expression on monocytes is also positively associated with the level of ALT in patients with CHB, indicating its role in disease progression[49]. Concordantly, Tim-3 expression is increased on monocytes from patients with HCV infection and is positively correlated with IL-17 levels in $CD4^+$ T cells, thus promoting Th17 cell accumulation. However, blocking Tim-3 on monocytes restores the balance of IL-12, IL-23 and IL-17 signaling *via* the STAT3 pathway[50,51].

Tim-3 and DCs

As the most potent antigen-presenting cells (APCs), DCs play a critical role in the innate immune response and greatly affect CHB progression. In particular, DCs control HBV recognition *in vivo*. Accumulating evidence has demonstrated that compared to DCs from healthy controls, DCs from patients infected with HBV exhibit impaired function[52,53], yet the reasons for this impairment remain unclear. Researchers have focused on developing a curative DC vaccine that activates the immune response through rescuing the function of DCs to treat HBV.

Constitutive expression of Tim-3 can be observed on DCs and may be positively associated with DC activation. Accordingly, stimulating Tim-3 with Gal-9 promotes TNF-α synthesis and secretion in cultured DCs[54]. Furthermore, using Gal-9 to activate Tim-3 signaling in tumor-bearing animal models can enhance the number of mature DCs and aid in the anti-tumor immune response[55]. However, some researchers have obtained opposing results, suggesting that Tim-3 might also be a negative regulator of DCs[56]. Compared with Tim-3-deficient bone marrow-derived DCs (BMDCs), $Tim\text{-}3^+$ BMDCs exhibit an impaired function phenotype. In particular, the latter cells showed a much poorer capacity to produce cytokines such as IFN-β1, IFN-α and IL-6[57]. The regulatory effect of Tim-3 on DCs is intermittent; many other factors, such as different pathogens and different ligands of Tim-3, can modulate the regulatory effect of Tim-3 on DCs.

In 2014, Ma *et al*[22] first engineered a live-attenuated HCV vaccine to stimulate immune cells. These researchers found that DCs isolated from healthy individuals demonstrated enhanced antigen presentation ability after stimulation, whereas DCs isolated from patients infected with HCV showed diminished responses after stimulation. Furthermore, blocking Tim-3 substantially rescued the function of DCs, indicating that Tim-3 may inhibit DC maturation. The effect of Tim-3 on DCs in HBV infection remains to be clarified; however, these findings observed in HCV infection may provide some clues[22].

Tim-3 and NK/NKTs

Over half of all liver lymphocytes are innate immune lymphocytes, and NKs account for the majority of these cells. Moreover, the percentage of NKs in the liver is approximately five-fold greater than that in spleen or blood, further indicating their important roles in liver diseases. Indeed, accumulating evidence has indicated the critical roles of NK cells in HBV-related diseases. The number and activity of circulating NKs are remarkably decreased in patients with CHB[58-60], and impaired NK function leads to persistent HBV infection and HCC tumorigenesis[61].

Tim-3 also appears to have conflicting effects on NKs. In some reports, the Tim-3-Gal-9 interaction has been shown to enhance IFN-γ production and Tim-3 has been described as a marker of fully mature NKs[62], whereas in other reports, for example, during HIV infection, Tim-3 has been found to inhibit the function of NKs, weakening the NK cell-mediated anti-viral immune response[63,64].

Our group first revealed the regulatory role of Tim-3 expression on NK cells in HBV infection. Increased Tim-3 expression on NK cells was detected in patients with CHB and in HBV-transgenic mice. In addition, Tim-3 expression on NK cells was positively correlated with the serum ALT levels in patients with CHB. Blockade of the Tim-3/Gal-9 interaction induced increased cytotoxicity and up-regulated IFN-γ production in both NKs from patients with CHB and in NK92 cell lines, strongly suggesting that Tim-3 plays negative roles in NKs during HBV[43]. However, the role of Tim-3 appears to be more complicated in HCV infection. Some studies have demonstrated that Tim-3 expression is elevated in NKs and that Tim-3 over-expression tends to result from down-regulated miR-155 in NKs during HCV infection, similarly to chronic HBV infection. Blocking Tim-3 can rescue the function of NKs, whereas reconstituting miR-155 can down-regulate Tim-3[65]. However, a recent study has reported the opposite results. Golden-Mason *et al*[66] have analyzed Tim-3 expression on NKs, demonstrating not only elevated expression of Tim-3 on these cells but also a positive correlation between Tim-3 and NK activity. These $Tim\text{-}3^+$ NKs also showed a stronger response to IFN-α stimulation and exhibited more intense killing activity[66]. Thus, the role of Tim-3 on NKs requires additional studies.

NKT-like cells, defined as $CD3^+CD16^+CD56^+$ cells, refer to a small population of T cells that co-express NK markers, for example, NK1.1 and CD56. If activated, these cells produce abundant pro-inflammatory cytokines and anti-inflammatory cytokines, including IFN-γ, MCP-1, and IL-4. Similarly to its expression on

monocytes and NKs, Tim-3 over-expression is also observed on NKT-like cells and is positively associated with the level of ALT in patients with CHB. Moreover, ACLF may further elevate Tim-3 expression[49].

Above all, elevated Tim-3 expression is observed in innate immunocytes and exerts a suppressive effect on their function during HBV infection (Figure 1). However, the role of Tim-3 in innate immune cells is complicated and requires further study.

TIM-3 POLYMORPHISMS AND HBV INFECTION

In 2012, Chinese scientists examined polymorphisms of the Tim-3 gene in a population of 712 individuals. Among these individuals, 182 represented healthy controls, and the others were patients with HBV-related liver diseases. The *Tim-3*-1541C/T, -1516G/T, -882C/T, -574G/T and +4259T/G polymorphisms were examined and analyzed, and the results showed that allele T-containing genotypes (GT+ TT), allele T and the allele T-containing haplotype (CTCGT) of the -1516G/T polymorphism occur more often in patients with CHB. The allele T-containing genotypes and allele T of -1516G/T are also associated with lymph node metastasis and tumor grade of HCC[67]. Other researchers have identified 2 other single nucleotide polymorphisms, rs31223 and rs246871, which correlate with the progression of HBV-induced liver disease. The minor allele "C" in rs31223 represents an increased chance of sero-clearance of HBsAg, whereas the genotype "CC" in rs246871 suggests an increased likelihood of developing HBV-related HCC. Furthermore, the haplotype blocks CGC* and TGC* strongly correlate with serum HBsAg sero-clearance, whereas CAT*, CGT*, TAC* and TGT* tend to be markedly correlated with HBV-induced HCC[68]. In accordance with their containment functions in negatively regulating immunity, polymorphisms of *Tim-3* and *PD-1* may differentially and interactively predispose individuals to HBV-related liver disease progression. The combined carriage of *PD1*+8669 AA/*TIM3* -1516 GT or TT shows a higher frequency in patients with cirrhosis than in patients without cirrhosis. Patients with HCC also have a higher frequency of this combined carriage than do patients with cirrhosis[69]. Together, these findings suggest that *Tim-3* polymorphisms may affect disease susceptibility and HCC traits associated with HBV infection.

TIM-3 AND HCC

The relationship between tumors and Tim-3 has been studied for many years, and Tim-3 may become the next major target in the treatment of cancer. In several cancer models, including breast cancer, colon cancer and melanoma, Tim-3 is induced in tumor-infiltrated lymphocytes and appears to mark exhausted $CD8^+$ T cells because $PD\text{-}1^+Tim\text{-}3^-CD8^+$ cells remain able to produce bona fide cytokines such as IFN-γ, IL-2 and TNF-α[70]. Here, we focus on the role of Tim-3 in liver cancer and discuss whether it can be regarded as a novel drug target in the treatment of liver cancer.

Chronic HBV infection is one of the most important risk factors for HCC, accounting for up to 54% of HCC cases worldwide, and this percentage is even higher in China. Accumulating evidence has supported the hypothesis that Tim-3 plays roles in HCC, particularly by modulating the tumor microenvironment. For instance, Tim-3 expression is elevated on $CD4^+$ and $CD8^+$ T cells infiltrating tumor tissues compared to those cells infiltrating the adjacent tissues, and $Tim\text{-}3^+$ T cells exhibit a senescence phenotype. Furthermore, the number of $Tim\text{-}3^+$ tumor-infiltrating cells is negatively correlated with patient survival, and Tim-3/Gal-9 signaling induces T cell senescence. Kupffer cells (KCs) have the highest Gal-9 expression, and $Tim\text{-}3^+$ T cells and $Gal\text{-}9^+$ KCs show a co-localization pattern in HCC. Blocking Tim-3/Gal-9 signaling re-activates tumor-infiltrating T cells, which display increased T cell proliferation and enhanced cytokine production. Moreover, in the HCC microenvironment, IFN-γ secreted by tumor-infiltrating T cells stimulates Gal-9 expression on APCs[71].

Tumor-associated macrophages (TAMs) are a major component of the tumor microenvironment and play a critical role in promoting tumor progression. Recently, our laboratory has discovered the important role of Tim-3 in TAM polarization in HCC. Specifically, elevated Tim-3 expression is negatively associated with tumor grade and patient survival. Moreover, ectopic expression of Tim-3 induces altered M2 activation, with a phenotype that promotes tumor development[44]. Our results further emphasize the critical role of Tim-3 as a new component in HCC progression.

In fact, Tim-3 is not expressed only in immune cells; our *in vivo* and *in vitro* experiments both demonstrated that Tim-3 is also expressed in HCC cells and that Tim-3 serves as an oncoprotein in these cells (unpublished data). These new data suggest that Tim-3 may have other functions in addition to immune inhibition.

HCC remains refractory to current chemotherapeutic drugs and generally is associated with a poor prognosis. Moreover, many patients with HCC cannot receive local ablative or surgical interventions because HCC is frequently diagnosed at an advanced stage. Recently, cancer immunotherapy has attracted substantial attention, and immune checkpoint blockade has achieved great success in many clinical trials. Although blockade of CTLA-4 and PD-1 has shown objective responses in several cancers, some issues still remain. For example, some patients have been shown to be non-responders to immunotherapies that target CTLA-4 and PD-1 in various clinical trials[72,73]. In this context, the discovery of novel immune checkpoints is

urgently required, and Tim-3 is a potential candidate. In 2010, Sakuishi reported that combined inhibition of Tim-3 and PD-1 demonstrated greater inhibition of tumor growth than PD-1 inhibition alone. Specifically, half of all tumor-bearing mice treated with combined blockade of Tim-3 and PD-1 displayed complete tumor regression, suggesting the crucial role of Tim-3 in tumor progression[74]. However, additional studies are required to demonstrate the therapeutic role of Tim-3 blockade in HCC treatment.

CONCLUSION

During HBV infection, Tim-3 expression is elevated on both adaptive and innate immune cells. This increased Tim-3 expression inhibits the anti-viral immune response, indicating that Tim-3 is a potential target for controlling HBV infection (Figure 1). However, several critical questions remain to be clarified. For example, how does HBV infection induce ectopic Tim-3 expression in different types of immune cells? What are the downstream signaling pathways promoted by Tim-3 in both adaptive and innate immune cells? Moreover, the regulatory role of Tim-3 in the innate immune response remains unclear, and Tim-3 may possess dual functions depending on the specific context. Therefore, we cannot be blindly optimistic to the potential of Tim-3 as a drug target for controlling chronic infection and HCC.

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Chronic pancreatitis: A diagnostic dilemma

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Abstract

Typical clinical symptoms of chronic pancreatitis are vague and non-specific and therefore diagnostic tests are required, none of which provide absolute diagnostic certainly, especially in the early stages of disease. Recently-published guidelines bring much needed structure to the diagnostic work-up of patients with suspected chronic pancreatitis. In addition, novel diagnostic modalities bring promise for the future. The assessment and diagnosis of pancreatic exocrine insufficiency remains challenging and this review contests the accepted perspective that steatorrhea only occurs with > 90% destruction of the gland.

Key words: Pancreatitis, chronic; Diagnosis; Exocrine pancreatic insufficiency; Pancreatic enzyme replacement therapy; Malabsorption

Core tip: Chronic pancreatitis presents a diagnostic challenge, especially in early disease. This paper summarizes the available diagnostic modalities as well as the most recently-published diagnostic guidelines. It is widely accepted that the pancreas has excellent exocrine reserve. We review the original studies which have supported this principle and suggest an alternative interpretation of the data.

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INTRODUCTION

Defined as a chronic inflammatory disease of the pancreas characterized by irreversible morphological change and typically causing pain and/or permanent loss of function[1], chronic pancreatitis is beset by destruction of healthy pancreatic tissue and the development of fibrous scar tissue. Gradual loss of exocrine and endocrine function ensues with clinical manifestations such as steatorrhea, abdominal pain, and diabetes. Current treatments can only provide temporary pain relief and manage complications, but are unable to halt or slow the advance of this disease[2]. The overall incidence of chronic pancreatitis in Europe is thought to be about 6-7 per 100000[3], and data suggest increasing incidence[4]. A study from the United Kingdom in the 1990's showed trends of rising incidence based on national statistics for admission[5]. Seven consecutive surveys from Japan have shown a definite increase in the prevalence of alcoholic chronic pancreatitis[6]. There are limited epidemiological data regarding the natural progression to chronic pancreatitis following an episode of acute pancreatitis. A study from North America[7] following over 7000 patients with an admission for acute pancreatitis found subsequent chronic pancreatitis in 12% of patients.

The majority of cases from western countries have been attributed to alcohol excess, although etiologies vary by region and country. The presenting symptom of most patients with chronic pancreatitis is abdominal pain, usually epigastric, dull and constant in nature. It is almost always localized in the upper half of the abdomen, from which it can radiate directly through to the back, or laterally around to the left or right flank. Initially the duration of pain is quite variable, ranging from several hours to several days, but as the disease progresses the attacks become more frequent and pain-free intervals shrink and vanish[8].

In some patients, chronic pancreatitis can be entirely silent, and in presentation patients may present with the sequelae of exocrine or endocrine insufficiency: steatorrhea, weight loss and diabetes. Less common initial presentations include biliary obstruction with recurrent episodes of mild jaundice, cholangitis, or vague attacks of indigestion[9]. Obstruction of the splenic vein by an inflamed tail of the pancreas can lead to left-sided portal hypertension, gastric varices and GI bleeding. Chronic pancreatitis and pancreatic cancer may present in a similar manner, making it difficult to distinguish between them[9].

Although chronic pancreatitis diagnosis may be suspected following presentation with suggestive symptoms, clinical presentation is usually insufficient for a firm diagnosis. In fact, a diagnosis of chronic pancreatitis is difficult to establish, especially in the early stages of disease. Typical symptoms such as weight loss, pain, steatorrhea, and malnutrition are vague and not specific to chronic pancreatitis. Therefore diagnostic tests of pancreatic structure and function are required - none of which provide absolute diagnostic certainly in the early stages.

The aim of this review is to: (1) summarize the available diagnostic modalities and the most recent diagnostic guidelines; (2) review emerging and novel diagnostic techniques; and (3) challenge the *status quo* regarding pancreatic exocrine insufficiency, specifically the accepted concept that steatorrhea occurs only with greater than 90% destruction of the gland.

DIAGNOSTIC TOOLS

There is no universally accepted diagnostic gold standard for chronic pancreatitis. While no one radiological, clinical or endoscopic tool can definitively diagnose this disease; there is an array of diagnostic instruments, which fall into four broad categories.

Histology

Histological features of chronic pancreatitis include parenchymal fibrosis, acinar atrophy, ductal distortion, and intraductal calcification[10,11]. Histological diagnosis is limited by a lack of consensus around grading for chronic pancreatitis[10]. Whilst histology is the most specific method of diagnosis, however it is rarely available and therefore proxy testing is required.

Radiological studies

Computed tomography: Computed tomography (CT) is a widely-used imaging modality and is an objective and reliable method of measuring pancreatic morphology. "Classical" diagnostic chronic pancreatitis findings on CT include atrophy, dilated pancreatic duct and pancreatic calcification (Figure 1A). While diagnosis of early chronic pancreatitis is not reliable, CT should nevertheless be performed in all patients to exclude a mass or gastro-intestinal malignancy[12]. In addition, CT may be used in the assessment of chronic pancreatitis-related complications, such as pseudocysts, pseudoaneurysm, duodenal stenosis and malignancy. CT should be performed using a non-contrast phase to identify calcifications followed by a "pancreas-protocol" contrast phase[13]. Overall, CT remains the best screening tool for detection of chronic pancreatitis and exclusion of other intra-abdominal pathology that may be indistinguishable from chronic pancreatitis based on clinical symptoms.

Magnetic resonance imaging, magnetic resonance cholangiopancreatography, and secretin-enhanced magnetic resonance cholangiopancreatography: Magnetic resonance imaging (MRI) is more sensitive than CT and is emerging as the initial radiological imaging modality of choice for the evaluation of chronic pancreatitis with unequivocal CT scans[12]. Magnetic resonance cholangiopancreatography (MRCP) allows for excellent

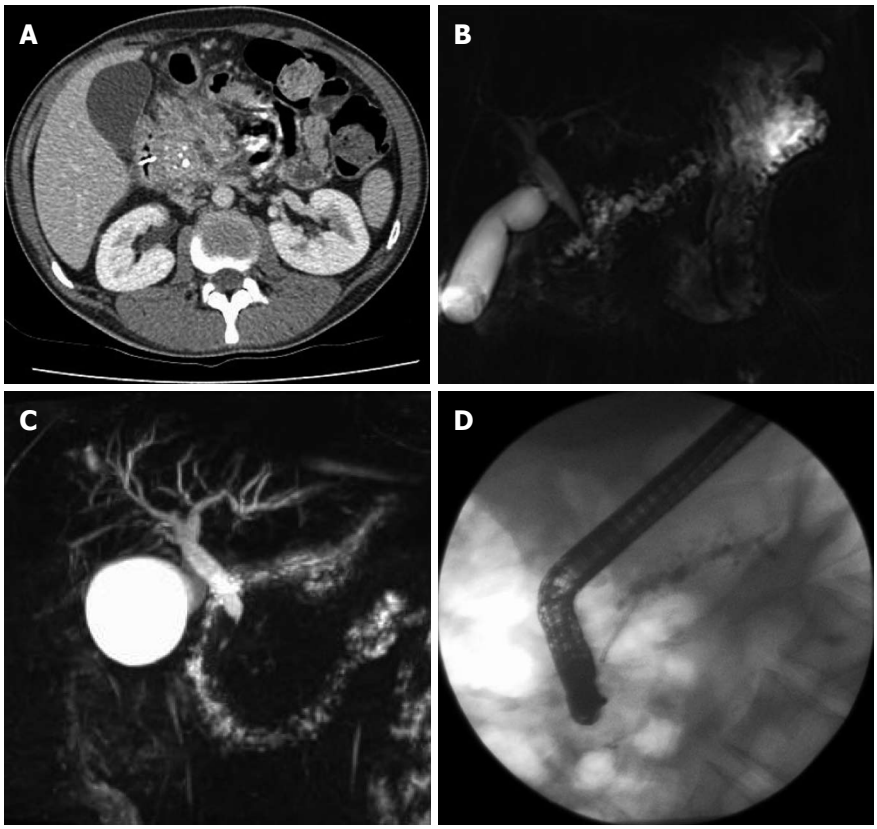

Figure 1 Computed tomography demonstrating enlarged head of pancreas with coarse calcification and a dilated main pancreatic duct (A), magnetic resonance cholangiopancreatography showing a tortuous, dilated pancreatic duct (B), inflammatory stricture of the distal common bile duct (C), endoscopic retrograde cholangiopancreatography showing a stent placed in a dilated pancreatic duct (D).

Table 1 Rosemont criteria for chronic pancreatitis

Parenchymal features	
Major A	Hyperechoic foci with stranding
Major B	Lobularity with honeycombing
Minor	Hyperechoic foci
	Lobularity
	Cysts
	Hyperechoic strands
Ductal features	
Major A	Calculi
Minor	Main pancreatic duct dilation
	Irregular main pancreatic duct contour
	Hyperechoic main pancreatic duct margin
	Dilated side branches

visualization of the pancreatic duct (Figure 1B and C), although visualization of the side branches is not good[14]. However, the addition of secretin enhancement provides even better visualization of abnormalities of the pancreatic duct and its branches, which may not have been evident on routine MRCP. Secretin stimulates fluid secretion in the ductal system, and increases the tonus of the sphincter of Oddi during the first 5 min, hindering the release of fluid through the papilla of Vater[14,15]. Therefore secretin increases the absolute volume of intraductal free water and fills the collapsed branches with fluid, thereby allowing the detection of mild ductal changes in mild disease that are not detectable using routine MRCP[15]. MRCP allows for similar visualization of the pancreatic duct as is afforded during the much more invasive endoscopic retrograde cholangiopancreatography (ERCP). MRCP also facilitates the diagnosis of complications of chronic pancreatitis such as biliary strictures (Figure 1C). Negatives associated with these modalities include limited access to MR time combined with the technical complexity of the test[16].

Endoscopic studies

Endoscopic ultrasound: Endoscopic ultrasound (EUS) provides close-proximity imaging of the entire pancreas and adjacent structures[17]. Although more invasive than CT and MRI/MRCP, EUS is the most sensitive imaging method for detecting minimal structural changes associated with chronic pancreatitis, and therefore is useful in minimal change or non-calcified chronic pancreatitis. The EUS-Rosemount criteria were published in 2009[18] as consensus-based criteria for EUS features of chronic pancreatitis (Table 1). Depending on the number of features identifiable, the following classification is applied: "consistent with chronic pancreatitis", "suggestive of chronic pancreatitis", "indeterminate for chronic pancreatitis", or "normal" (Table 2)[18]. It is still unresolved whether or not "indeterminate for chronic pancreatitis" refers to early-stage chronic pancreatitis[19]. The number of

Table 2 Endoscopic ultrasound diagnosis of chronic pancreatitis on the basis of Rosemont criteria

Consistent with chronic pancreatitis	1 major A feature + ⩾ 3 minor features or 1 major A feature + major B feature or 2 major A features
Suggestive of chronic pancreatitis	1 major A feature + < 3 minor features or 1 major B + ⩾ 3 minor features or ⩾ 5 minor features
Indeterminate for chronic pancreatitis	3 or 4 minor features, no major features or Major B feature alone with < 3 minor features
Normal	⩽ 2 minor features, no major features

EUS criteria for diagnosis varies between institutions, and in additions, intra-observer agreement among endosonographers is low, which is one of its greatest limitations[20]. Conwell and colleagues[17] showed that based on a gold standard pancreatic function test [an endoscopic, secretin-stimulated pancreatic function test (PFT)], 6 or more EUS criteria are needed to establish a definitive diagnosis of chronic pancreatitis. However, less than 6 EUS criteria may be associated with pancreatic secretory dysfunction, and so, EUS may not be an adequate screening modality for early chronic pancreatitis where there is an absence of significant parenchymal and ductal scarring[17].

ERCP: ERCP is considered a sensitive test for the diagnosis of chronic pancreatitis, having the ability to show dilation or stricture of the pancreatic duct and its branches, as well as early features of chronic pancreatitis[21]. ERCP provides therapeutic options, such as dilation, stone extraction, and stenting of the duct (Figure 1D). An additional benefit is the possibility of procuring pancreatic juice during ERCP[22]. The Cambridge criteria developed in 1984[23] allows the classification of chronic pancreatitis based on the number of ductal abnormalities found at ERCP. However, with the widespread availability of other non-invasive imaging modalities, ERCP should not be used for the diagnosis of chronic pancreatitis. ERCP is also limited by the fact that it does not allow evaluation of the pancreatic parenchyma[10]. Axial imaging (CT or MRCP) and EUS have replaced ERCP as a diagnostic tool and the principles of the Cambridge classification can be adapted to CT or MRCP.

Pancreatic function tests

Direct pancreatic function testing: PFTs for the testing of exocrine function may be classified as direct and indirect. Direct tests involve the stimulation of the pancreatic cells using secretagogues (secretin or cholecystokinin, CCK). These tests are invasive (requiring endoscopic procedures), expensive and tend not to be widely done outside of specialist centers. Sensitivity is high for direct PFTs in the diagnosis of late chronic pancreatitis, however lower (70%-75%) for early chronic pancreatitis. Direct PFTs have a long history (from the 1900s), and the original Dreiling tube method[24,25] (popularized at the University of Florida) and newer methods such as the endoscopic PFT (ePFT, developed at the Cleveland Clinic) are considered the nonhistological criterion standards for diagnosis of early chronic pancreatitis[2].

Indirect pancreatic function testing: The invasive nature of direct testing, along with the expense and unavailability of the tests, obligates indirect means of pancreatic function testing. Such tests include fecal elastase, fecal fat measurements and serum trypsinogen. The 3 d fecal fat collection test requires the collection of stool for a 72 h period following the ingestion of a precisely-known quantity of fat (100 g per day). Excretion of more than 7 g of fat in the stool per day is indicative of fat malabsorption, while loss of more than 15 g per day is considered severe fat malabsorption. However the 3 d fecal fat assessment is a cumbersome test for both patients and laboratory personnel, and is not routinely done. In general, indirect tests are moderately sensitive and specific for diagnosing advanced chronic pancreatitis, but less so for early disease. Pancreatic elastase-1 fecal elastase-1 (FE-1) is a human-specific enzyme that is not degraded during intestinal transit, is enriched 5-6 fold in the feces, and is therefore a test of pancreatic exocrine function. Benefits include the fact that patients do not have to consume a specific substrate (*i.e.*, fat) prior to testing, nor must they halt pancreatic enzyme replacement therapy. However whilst FE-1 is an adequate measure of severe exocrine impairment, it is not a good indicator of mild to moderate disease.

DIAGNOSTIC GUIDELINES FOR CHRONIC PANCREATITIS

American pancreatic association guidelines

At the 2011 meeting of the American Pancreatic Association, a chronic pancreatitis conference was held to develop a 3-part set of practice guidelines for this disease. The first part of these guidelines relates to diagnosis and was published in 2014[2]. The document, which represents the first US practice guidelines for chronic pancreatitis, defines the diagnostic evidence for CP as definitive, probable and insufficient based on current knowledge. The guidelines emphasize that without sufficient evidence, patients should not be mislabeled as having chronic pancreatitis, and it is better to err on the side of not labelling the patient with chronic pancreatitis, recommending longitudinal follow-up with serial imaging and physiological testing in unequivocal cases until definitive evidence is present. The guidelines propose a diagnostic algorithm which proceeds from non-invasive to a more invasive approach (Figure 2). Upon confirmed diagnosis, the guidelines recommend a comprehensive etiological/morphological and physiological characterization of chronic pancreatitis, and propose an associated nomenclature. This nomenclature recommends the following structure: chronic (TIGARO etiology-induced)

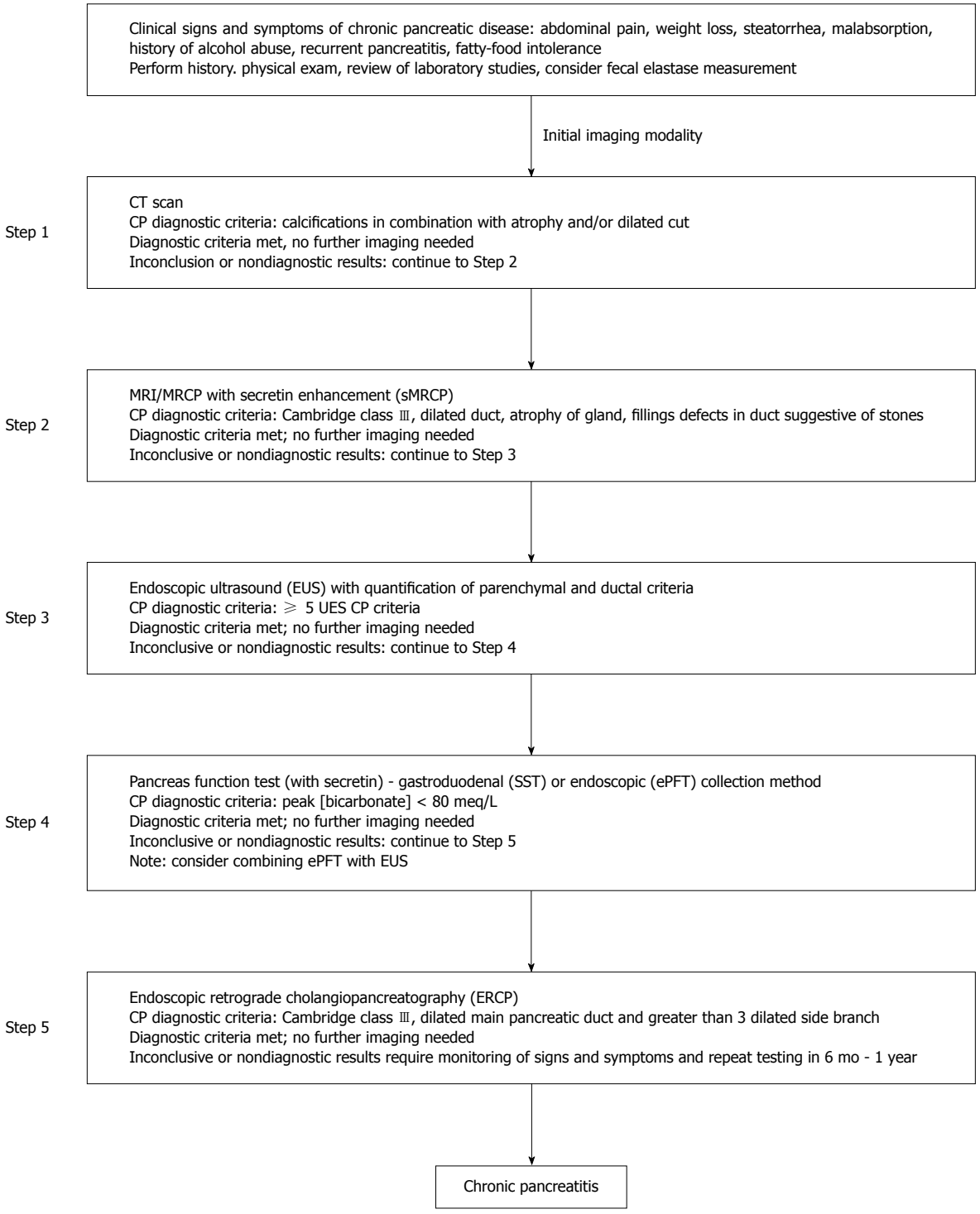

Figure 2 Step-wise algorithm approach to diagnosis of chronic pancreatitis. Step 1: Survey (data review, risk factors, CT-imaging); Step 2: Tomography (pancreas protocol CT scan, MRI/secretin-enhanced magnetic resonance cholangiopancreatography); Step 3: Endocopy [EUS (standard criteria)]; Step 4: Pancreas functioning (Dreiling, ePFT); Step 5: ERCP (with intent for therapeutic intervention). From Conwell *et al*[2]. CT: Computed tomography; MRI: Magnetic resonance imaging; EUS: Endoscopic ultrasound; ERCP: Endoscopic retrograde cholangiopancreatography.

pancreatitis + MANNHEIM/Cambridge imaging grade + physiological stage (where TIGARO is toxic-metabolic, idiopathic, genetic, autoimmune, recurrent and severe acute pancreatitis associated, obstructive). The document details the available evidence for 9 topics, giving Evidence-Based Medicine Statements for each. With the exception of the anatomic pathology topic, each statement is given a recommendation (strong/conditional) and the level of evidence is defined as strong/moderate/low. The proportion of "strong" *vs* "conditional" statements was roughly half and half. The intention of the group is to modify these guidelines with emerging evidence. The APA diagnostic guidelines are summarized in Table 3.

Table 3 A summary of the APA diagnostic guidelines (2014)

Topic		Level
Epidemiology and risk factors	Data on population-based estimates are emerging	C/L
	A small fraction of patients progress from AP to CP	C/M
	Alcohol/smoking are independent risk factors for CP. Both are associated with disease progression and their risks are likely multiplicative	S/H
	The spectrum of risk factors for CP has broadened	C/L
	Genetic discoveries are rapidly uncovering new susceptibility factors. Knowledge of gene-environment interactions may translate into new diagnostic and treatment paradigms	S/M
Pathological Definitions	CP is characterized by atrophy and fibrosis of the exocrine tissue with or without chronic inflammation	-
	Scarring of the parenchyma may be focal, patchy or diffuse	-
	Progressive fibrosis and atrophy may lead to exocrine insufficiency followed by endocrine insufficiency	-
	Autoimmune pancreatitis can mimic pancreas carcinoma	-
Ultrasound and CT	Ultrasound and CT are best for late findings of CP but are limited in the diagnosis of early or mild pancreatitis	C/M
	Intraductal pancreatic calcifications are the most specific and reliable sonographic and CT signs of CP	S/M
	CT is helpful for the diagnosis of complications of CP	S/M
	CT is helpful for the diagnosis of other conditions that can mimic CT	C/L
MRI imaging	Compared with ultrasound and CT, MRI is a more sensitive imaging tool for the diagnosis of CT	C/M
	Ductal abnormalities are very specific and reliable MRI signs of CP	C/L
	Signal intensity changes in the pancreas, seen on MRI, may precede ductal abnormalities and suggest early CT	C/L
	Stimulation of the pancreas using IV secretin may improve the diagnostic accuracy in the detection of ductal and parenchymal abnormalities seen on CT	C/L
Endoscopic ultrasound	The ideal threshold number of EUS criteria necessary to diagnose CP has not been firmly established, but the presence of 5 or more or 2 or less strongly suggests or refutes the diagnosis of CP	S/L
	The EUS features of CP are not necessarily pathologic and may occur as a normal aging, as a normal variant, or due to the nonpathologic asymptomatic fibrosis in the absence of endocrine/exocrine dysfunction	S/L
	The relatively poor IOA for EUS CP features limits the diagnostic accuracy and overall utility of the EUS for diagnosing CP	S/M
ERCP	ERP is rarely used for diagnostic purposes	S/M
	The correlation between the Cambridge criteria and histology is highest in advanced CP	S/M
	Multiple confounders limit the interpretation of ductal changes by Cambridge criteria	S/L
Indirect PFTs	Indirect PFTs generally are sensitive for steatorrhea and useful in quantifying the degree of exocrine insufficiency	C/L
	Indirect PFTs are moderately sensitive and specific for diagnosing advanced CP but are less so for diagnosing early CP	C/S
	The FE-1 assay, polyclonal assay more than monoclonal, can be limited in specificity, especially if the stool has is watery and/or in the presence of small bowel disease	C/L
	Faecal chymotrypsin may be useful in detecting compliance with exogenous pancreatic enzyme supplementation	C/L
	Faecal fat assays are sensitive for steatorrhea but are of limited utility due to the cumbersome nature of patient collection and laboratory handling of samples. In addition, strict adherence to dietary recommendations for several days is required	C/M
Direct PFTs	Direct PFTs have high sensitivity for detecting late CP, but lower sensitivity (70%-75%) for early CP	S/L
	The traditional secretin and CCK PFTs performed with the aortoduodenal tube pancreas fluid collection are highly accurate but require fluoroscopy for confirmation of tube placement and are not widely utilized	S/M
	The ePFT has good correlation with the traditional Dreiling PFT	S/M
Correlation of imaging and function with histology	As structural severity worsens in CP, exocrine function declines	S/M
	Both EUS and PFT results correlate with fibrosis in CP	C/L
	A combined approach (*e.g.*, EUS/ePFT) could improve detection of minimal change CP (MCCP)	C/L

Levels relate to level of recommendation (conditional; strong)/level of evidence (low; moderate; high). AP: Acute pancreatitis; CP: Chronic pancreatitis; CT: Computed tomography; MRI: Magnetic resonance imaging; IV: Intravenous; EUS: Endoscopic ultrasound; IOA: Inter-observer variability; PFTs: Pancreatic function tests; FE-1: Fecal elastase-1; CCK: Cholecystokinin; ePFT: Endoscopic PFT; MCCP: Minimal change chronic pancreatitis.

Other guidelines

Conwell and Bechien[12] devised an algorithm for the stepwise diagnosis of chronic pancreatitis. Using the most commonly available radiological and endoscopic tests, the algorithm progresses from a non-invasive to an invasive approach, starting with a clinical Survey, Tomography (imaging), Endoscopy, and finally Pancreatic function. The authors caution against mislabeling patients with a chronic pancreatitis diagnosis where they instead have a chronic abdominal pain syndrome with a remote history of procedure-induced pancreatitis.

In 2010, the Japanese Clinical Diagnostic criteria for chronic pancreatitis were published[26]. These criteria were intended to diagnose "early chronic pancreatitis", with the intention of preventing intractable disease by allowing early treatment. The diagnostic tool specifies that 2 of the following 4 items be present: repeated upper abdominal pain, abnormal pancreatic enzyme levels (serum or urine), abnormal pancreatic function, and on-going heavy alcohol ingestion (of > 80 g pure ethanol per day). These items, along with characteristic early findings by EUS imaging are said to be indicative of early chronic pancreatitis. According to this tool, more than 2 of the following EUS criteria are required for diagnosis (as well as at least one from the first 4 criteria: (1) lobulating with honeycombing; (2) lobulating without honeycombing; (3) hyperechoic foci with stranding; (4) stranding; (5) cysts; (6) dilated side branches; and (7) hyperechoic MPD margin. More

recently, reports of the Tissue Harmonic Echo mode on EUS have suggested that these modes can reveal details of abnormalities of early chronic pancreatitis and might therefore contribute to a definite diagnosis in the early stages of disease[27].

Guidelines were published in 2010 by the Hepato-Pancreatico-Biliary Association of South Africa, along with the South Africa Gastroenterology Society which summarized the diagnostic tools for chronic pancreatitis[28]. The authors suggested that the choice of imaging study should be based on the available technology, the available skills and the invasive nature of the investigation. They emphasize the limitations of PFTs in the diagnosis of chronic pancreatitis, stressing that PFTs alone do not distinguish chronic pancreatitis from pancreatic exocrine insufficiency (PEI) without chronic pancreatitis.

Emerging diagnostic techniques

Engjom *et al*[16] in 2015 described a technique which evaluated ultrasonography of the fluid in the descending duodenum and Wirsung duct, after secretin stimulation, as a measure of pancreatic fluid flow. Using both chronic pancreatitis and cystic fibrosis patients, those with pancreatic exocrine insufficiency (Defined as FE-1 < 100 μg/g, or peak bicarbonate concentrations of > 80 meq/L) were compared to healthy controls. Ultrasonography gave precise measurement of the volume transported in the descending duodenum and Wirsung duct after secretin stimulation. The authors identified subjects with severe pancreatic output failure compared to healthy controls with good diagnostic accuracy.

EUS elastography is a recently described diagnostic tool which quantitatively analyses pancreatic tissue consistency. This method enables areas with varying elasticities to be differentiated within the pancreas. The principle of elastography is based on the assumption that compression of a target tissue by an echo-endoscopic probe creates a strain that differs according to the hardness and softness of the tissue. During the procedure, elastography is shown in real time as transparent colour images[29]. Quantitative elastography therefore allows for the quantitative assessment of fibrosis in chronic pancreatitis. In quantitative elastography, the tissue stiffness is measured in the targeted area [region of interest (ROI) A] and outside the targeted area in a region representing normal tissue (ROI B). Thereafter, the strain ratio value is calculated as the quotient B/A. One study[30] on EUS elastography in chronic pancreatitis found excellent concordance between EUS criteria for chronic pancreatitis and strain ratio, and reported a diagnostic accuracy of 91%. A further study from this group[31] evaluated whether EUS-elastography can predict PEI in chronic pancreatitis. Comparing elastography to the C-mixed triglyceride breath test, pancreatic strain ratio was higher in those with PEI than with a normal breath test. The probability of PEI was 87% with a strain ratio of greater than 4.5, and could therefore be considered for pancreatic enzyme therapy, even in the absence of any pancreatic function test. The relationship between pancreatic morphology and exocrine function is discussed in the following section.

The occurrence of nutrition deficiencies in chronic pancreatitis has recently been suggested by Lindkvist *et al*[32] as an indicator of PEI. One hundred and fourteen patients had a chronic pancreatitis diagnosis based on endoscopic ultrasonography or MRI, and PEI was investigated by the ^{13}C-mixed triglyceride breath test. They found that serum nutritional markers were able to predict PEI with reasonably high sensitivity and specificity.

RELATIONSHIP BETWEEN PANCREATIC DESTRUCTION AND FAT MALABSORPTION: CHALLENGING THE *STATUS QUO*

PEI is the reduction in pancreatic enzyme activity in the intestinal lumen to a level that is below the threshold required to maintain normal digestion[33]. It is widely believed that the pancreas has a large exocrine reserve. This is largely due to a landmark study published in 1973 by DiMagno *et al*[34], which studied the relationship between malabsorption and lipase secretion of the pancreas. They reported confirmation "that 90% of the gland must be functionally destroyed or obstructed before steatorrhea or creatorrhea occurs", and that "fat digestion is not clearly impaired until lipase outputs are decreased to about 10% of normal". These findings were based on a comparison of 17 patients with chronic pancreatitis and 33 healthy controls. Total enzyme output was measured in response to duodenal perfusion with essential amino acids and intravenous cholecystokinin-pancreaozymin in patients and controls. Values were expressed as a percentage of normal, which was derived from the healthy controls. While the study was well-conducted, the data interpretation was open to debate. The low sample size of 17 was itself not necessarily a limitation, as few subjects are required to show statistical significance where there is a large effect. However, critically, 16/17 patients had poor pancreatic function (defined by lipase secretion < 10% of normal), therefore, the authors can only conclude that those with poor pancreatic secretory function (and presumably severe disease) suffer fat excretion consistent with steatorrhea (> 7 g per day) (Figure 3). The one patient with high percentage lipase secretion happened to have normal fat absorption; however, this sole patient does not provide enough evidence that those with greater than 10% pancreatic function have normal fat excretion. Moreover, among

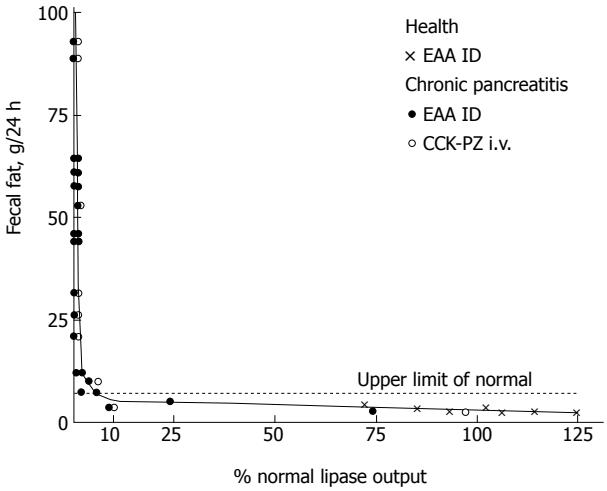

Figure 3 Relation of lipase outputs per 24 h to fecal fat excretion in healthy subjects and patients with chronic pancreatitis. Values above the horizontal dashed line denote steatorrhea (> 7 g per 24 h). The shaded area represents lipase outputs less than 10 percent of normal. From DiMagno *et al*[34]. Copyright © 2015 Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society.

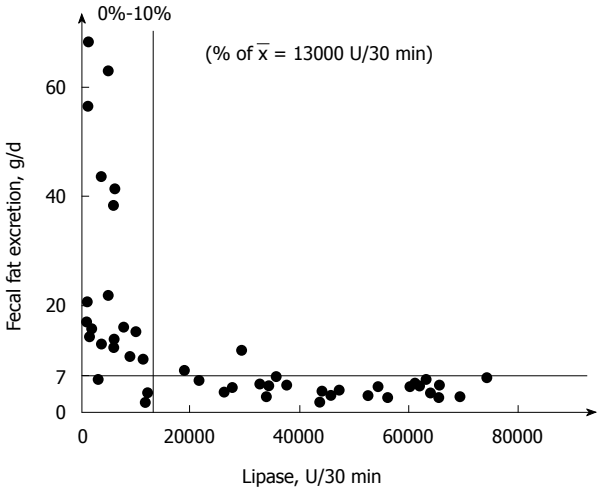

Figure 4 Relation of lipase output to fecal fat excretion in 47 patients with exocrine insufficiency. Reprinted with permission from Lankisch *et al*[35]. Copyright © 2015 Karger Publishers, Basel, Switzerland.

the majority that did have low lipase secretion (< 10% normal lipase output), the range of fat loss per day was extraordinarily broad. Those with severely reduced lipase output secondary to chronic pancreatitis exhibited fat malabsorption ranging from mild (about 10 g/24 h), to very severe (almost 100 g/24 h), an enormously broad range in clinical terms.

Lankisch *et al*[35] conducted a similar, larger study in 1986 (n = 47 chronic pancreatitis patients) with a broader range of exocrine impairment. Figure 4 displays the relationship between lipase output and fecal fat excretion from this study. Consistent with the DiMagno study, most patients with < 10% lipase excretion had steatorrhea. However not all did; three patients with < 10% lipase excretion had normal fecal fat excretion. And similarly consistent with the DiMagno study there was a remarkably broad variation in fecal fat excretion for those with lipase secretion of < 10 g/d, ranging from normal to greater than 60 g/d. Unlike the DiMagno study, the Lankisch study included subjects with moderately impaired lipase secretion, and of those patients, two (7.7%) had steatorrhea. Furthermore, 16.2% and 15.6% of patients with moderate impairment of amylase and trypsin respectively exhibited steatorrhea (graphs not shown). Therefore steatorrhea was not limited to those with extreme pancreatic damage.

Various studies quantifying lipase excretion in chronic pancreatitis have been published. Conwell *et al*[36] investigated peak lipase concentration by means of a CCK-stimulated pancreatic function test in 19 healthy volunteers and 18 patients with chronic pancreatitis. They found that lipase concentration in duodenal fluid was markedly lower in those with chronic pancreatitis compared to controls; 50% in mild chronic pancreatitis, 23% in moderate chronic pancreatitis and 13% in severe chronic pancreatitis. Mizuno *et al*[37] found that lipase output in severe disease and mild disease was 10% and 60% respectively. Ideally, the studies conducted by DiMagno *et al*[34] and Lankisch *et al*[35] should be repeated on an adequately large number of subjects with a broad spectrum of lipase outputs. Intuitively one would expect a linear relationship between lipase secretion and fat excretion. The seemingly non-linear relationship suggested by DiMagno *et al*[34] has been contradicted in an artificial model of steatorrhea (induced by lipase-inhibitor orlistat). They showed a linear and positive correlation between lipolysis inhibition and fat excretion levels[38].

The appearance of sufficient pancreatic exocrine function (until > 90% gland destruction) may be in part due to the secretion and action of gastric lipase. There is an element of compensation by gastric lipase in chronic pancreatitis patients with advancing disease, essentially giving the illusion of adequate pancreatic exocrine function. As well as evidence of increased secretion of gastric lipase in severe (*vs* mild) chronic pancreatitis and healthy controls, gastric lipase is also more stable in severe chronic pancreatitis due to an increase in the specific activity of the enzyme[39]. Gastric lipase has higher specific activity at acidic pH values, and those with chronic pancreatitis are known to have more acidic small intestine contents than normal patients[40] (due to reduced bicarbonate excretion). Hence, this provides another reason to revisit data from early studies examining an association between lipase excretion and fecal fat loss, as the clinically relevant contribution of gastric lipase had not been considered. Indeed the contribution of gastric lipase may partially explain the remarkably broad range of fecal fat excretion in patients with pancreatic lipase excretion of < 10% normal (Figures 3 and 4).

The 1973 paper by DiMagno *et al*[34], along with the

1986 paper by Lankisch *et al*[35], appear to be the only studies that have examined exocrine insufficiency and fat excretion in this manner. The study by DiMagno in particular has greatly influenced understanding and practice in PEI and is widely cited as evidence of adequate exocrine function until almost total pancreatic destruction. Therefore it is possible that PEI is ignored, disregarded and untreated in all but the most morphologically severe patients. In fact, a study from The Netherlands[41] found that a considerable number of patients with PEI were undertreated, with 70% of subjects reporting ongoing steatorrhea-related symptoms, and 42% still suffering weight loss. Undertreatment may result in PEI-related abdominal symptoms, weight loss, muscle depletion, nutrient deficiency[42,43], and deficiency-related complications, including osteoporosis[44,45] and premature fragility fracture[46,47].

CONCLUSION

Diagnosis of chronic pancreatitis continues to present a clinical challenge; however recent guidelines have brought much need direction and clarity to this endeavor. In the assessment of pancreatic exocrine function, the traditional viewpoint that steatorrhea does not occur until > 90% of the pancreas is destroyed is still often quoted and accepted. We have challenged this perspective by revisiting the old data and suggesting an alternative interpretation. The perception that the pancreas has excellent exocrine reserve needs to be reconsidered, not least due to the potential disregard for PEI and resultant delays in establishing appropriate and adequate enzyme therapy that are likely to occur if this unsound principle continues to be accepted.

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ORIGINAL ARTICLE

Basic Study

Mutation analysis of 13 driver genes of colorectal cancer-related pathways in Taiwanese patients

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Abstract

AIM: To investigate the driver gene mutations associated with colorectal cancer (CRC) in the Taiwanese population.

METHODS: In this study, 103 patients with CRC were evaluated. The samples consisted of 66 men and 37 women with a median age of 59 years and an age range of 26-86 years. We used high-resolution melting analysis (HRM) and direct DNA sequencing to characterize the mutations in 13 driver genes of CRC-related pathways. The HRM assays were conducted using the LightCycler® 480 Instrument provided with

the software LightCycler® 480 Gene Scanning Software Version 1.5. We also compared the clinicopathological data of CRC patients with the driver gene mutation status.

RESULTS: Of the 103 patients evaluated, 73.79% had mutations in one of the 13 driver genes. We discovered 18 novel mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53* that have not been previously reported. Additionally, we found 16 *de novo* mutations in *APC*, *BMPR1A*, *MLH1*, *MSH2*, *MSH6*, *MUTYH* and *PMS2* in cancerous tissues previously reported in the dbSNP database; however, these mutations could not be detected in peripheral blood cells. The APC mutation correlates with lymph node metastasis (34.69% *vs* 12.96%, P = 0.009) and cancer stage (34.78% *vs* 14.04%, P = 0.013). No association was observed between other driver gene mutations and clinicopathological features. Furthermore, having two or more driver gene mutations correlates with the degree of lymph node metastasis (42.86% *vs* 24.07%, P = 0.043).

CONCLUSION: Our findings confirm the importance of 13 CRC-related pathway driver genes in the development of CRC in Taiwanese patients.

Key words: Colorectal cancer; Driver gene; Colorectal cancer-related pathway; Mutation; High-resolution melting analysis

Core tip: In Taiwan, colorectal cancer (CRC) has had the highest incidences among cancers recently. In a study of 103 patients with CRC, we identified 18 novel mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53*. We assessed the frequency of non-pathological somatic mutations during oncogenesis, which has not been explored before. Our results indicated 16 *de novo* mutations that have been previously described in a public database and were detected in cancerous tissues only, but not in the patient's blood cells. We suggest these mutation sites may belong to a frequent mutational hotspot in both germline and cancerous tissues.

Chang YC, Chang JG, Liu TC, Lin CY, Yang SF, Ho CM, Chen WT, Chang YS. Mutation analysis of 13 driver genes of colorectal cancer-related pathways in Taiwanese patients. *World J Gastroenterol* 2016; 22(7): 2314-2325 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2314.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2314

INTRODUCTION

Colorectal cancer (CRC) is one of the major causes of mortality and morbidity in Western and in Asian countries. In 2014, an estimated 136830 new cases were diagnosed, and 50310 deaths were due to CRC, making it the third most common cancer among men and women in the United States[1]. The lifetime risk of CRC is 6%, and the average age at diagnosis is 66 years old in the United States[2]. CRC has also become the third leading cause of cancer-related death in the Taiwanese population[3].

Inherited CRCs can be attributed to hereditary nonpolyposis CRC (HNPCC), familial adenomatous polyposis (FAP), and closely related variant syndromes[4]. Approximately 15%-30% of the patients may fall into this category, and their first- or second-degree relatives may have CRC[5]. New or *de novo* germ-line mutations of adenomatous polyposis coli (*APC*) occur in approximately 25% of FAP cases. The lifetime incidence of CRC in untreated FAP patients is approaching 100%[6]. The most common germline *APC* mutations are located at codons 1061 and 1309[7]. HNPCC is the result of germline mutations in DNA mismatch repair (*MMR*) genes, including mutL homolog 1 (*MLH1*), mutS homolog 2 (*MSH2*), mutS homolog 6 (*MSH6*) and PMS1 homolog 2 (*PMS2*), with mutations in *MLH1* and *MSH2* being more common than those in other *MMR* genes[8]. Juvenile polyposis syndrome is caused by mutations in the bone morphogenetic protein receptor, type 1A (*BMPR1A*) or SMAD family member 4 (*SMAD4*) tumor suppressor genes[9]. Cowden syndrome is associated with mutations in phosphatase and tensin homolog (*PTEN*)[10]. Homozygous mutations in the base excision repair (BER) pathway gene mutY DNA glycosylase (*MUTYH*) cause *MUTYH*-associated polyposis syndrome, and heterozygous *MUTYH* mutations are found in some cases of familial CRC[11].

Sporadic CRCs account for approximately 70%-85% of all cases, and these patients have no distinguishable genetic risk factors. The development of sporadic CRC is probably as a result of diet, lifestyle, and environmental factors as well as somatic mutations[12]. Sporadic CRCs have more biological variables compared with hereditary CRCs[13]. Chromosomal instability (CIN), microsatellite instability and CpG island methylator phenotype pathways are the major genetic mechanisms responsible for sporadic CRCs[14]. The CIN pathway implies the progression from adenoma to carcinoma. This pathway suggests a stepwise pattern of mutational inactivation of tumor suppressor genes, such as the *APC* and tumor protein p53 (*TP53*), and the activation of oncogenes, such as Kirsten rat sarcoma viral oncogene homolog (*KRAS*)[15]. Most sporadic CRCs (70%-80%) have *APC* somatic mutations, and the mutations appear to be enriched in the mutation cluster region (MCR, codons 1309 to 1450)[7]. Approximately 40% of CRCs have *KRAS* mutations, and almost all of these mutations are located at codons 12, 13 or 61[16,17]. The MSI pathway

is characterized by the inactivation of the MMR genes such as *MLH1*. Inactivation of MMR genes occurs either through *MLH1* promoter hypermethylation or point mutations in one of the MMR genes. *De novo* germline mutations or somatic mutations in MMR genes account for a small number of sporadic CRCs[8]. The Serrated Pathway is characterized by the presence of a mutation in the oncogene v-raf murine sarcoma viral oncogene homolog B (*BRAF*) and the hypermethylation of other genes[18]; 3%-13% of CRC patients have a mutation in the *BRAF* gene[19].

Multiple previous reports have revealed that several critical genes and pathways are important in the initiation and progression of CRC, these include WNT, RAS-MAPK, PI3K, TGF-β, P53, and DNA MMR pathways[20]. The Cancer Genome Atlas Network project has identified numerous recurrently mutated genes[21].

The aim of our study was to assess the genes known to be implicated in CRC and to compare the clinicopathological data with the molecular genetic profiles of the tumors. We used a high resolution melting (HRM) technique and direct DNA sequencing to analyze the *APC* exons 1-14 and part of exon 15, *BRAF* exon 15, *KRAS* exon 2, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (*PIK3CA*) exon 9 and 20, the complete coding region of *BMPR1A*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PMS2*, *PTEN*, *SMAD4* and *TP53* in CRCs from 103 Taiwanese patients.

MATERIALS AND METHODS

DNA samples

One hundred and three colorectal adenocarcinomas were collected and analyzed. All samples were tested for sporadic and familial genetic changes in known CRC related genes (*APC*, *BMPR1A*, *BRAF*, *KRAS*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PIK3CA*, *PMS2*, *PTEN*, *SMAD4* and *TP53*). DNA was extracted using a commercially available kit (GE Healthcare, Little Chalfont, UK), following the manufacturer's recommendations. After extraction, DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington DE, United States). This study was approved by the Institutional Review Board of the China Medical University Hospital.

HRM technique

To assess *APC*, *BMPR1A*, *BRAF*, *KRAS*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PIK3CA*, *PMS2*, *PTEN*, *SMAD4* and *TP53* mutations, we performed HRM of small amplicons using a LightCycler® 480 Instrument (Roche Diagnostics, Roche Instrument Center AG, Rotkreuz, Switzerland) in tumor samples from CRC patients. The primers used for HRM analysis are shown in Supplementary material. The amplifications were performed in 10 μL volumes containing 10 ng of genomic DNA, 0.25 μmol/L primers, 2.5 mmol/L $MgCl_2$ and 5 μL 2X LightCycler® 480 High Resolution Melting Master (reference 04909631001, Roche Diagnostics) buffer. Polymerase chain reaction cycling included an initial denaturation at 95 ℃ for 10 min followed by 45 cycles of 15 s at 95 ℃, 15 s at 60 ℃, and 15 s at 72 ℃. The melting program included three steps: denaturation at 95 ℃ for 1 min, renaturation at 40 ℃ for 1 min, and a subsequent melting cycle that consists of a continuous fluorescent reading from 60 to 90 ℃ at a rate of 25 acquisitions per ℃.

Gene scanning

Gene scanning analysis of the data using Gene Scanning Software consisted of three steps: (1) normalization of melting curves, which involved setting the initial fluorescence equal to 100% and the remaining fluorescence signal after DNA dissociation to 0%; (2) shifting of the temperature axis of the normalized melting curves to the point where the entire double-stranded DNA was completely denatured; and (3) generation of difference plots, allowing capture of the melting profile difference between the reference sample curves and the test samples.

Direct sequencing

After HRM analysis, the samples were purified using the PCR-M™ clean up system (VIOGEN, Sunnyvale CA, United States). The sequence reaction was performed using 1 μL of the purified PCR product, 2.5 μmol/L of one of the PCR primers and 1 μL ABI PRISM terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA) in a final reaction volume of 10 μL. The samples were sequencing using a 25-cycle PCR program (denaturation at 96 ℃ for 10 s, annealing at 50 ℃ for 5 s and elongation at 60 ℃ for 4 min). The sequencing detection was performed using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

Due to its size (2545 nucleotides) it was costly and time consuming to screen *MSH6* exon 4 using HRM; thus, direct DNA sequencing was performed.

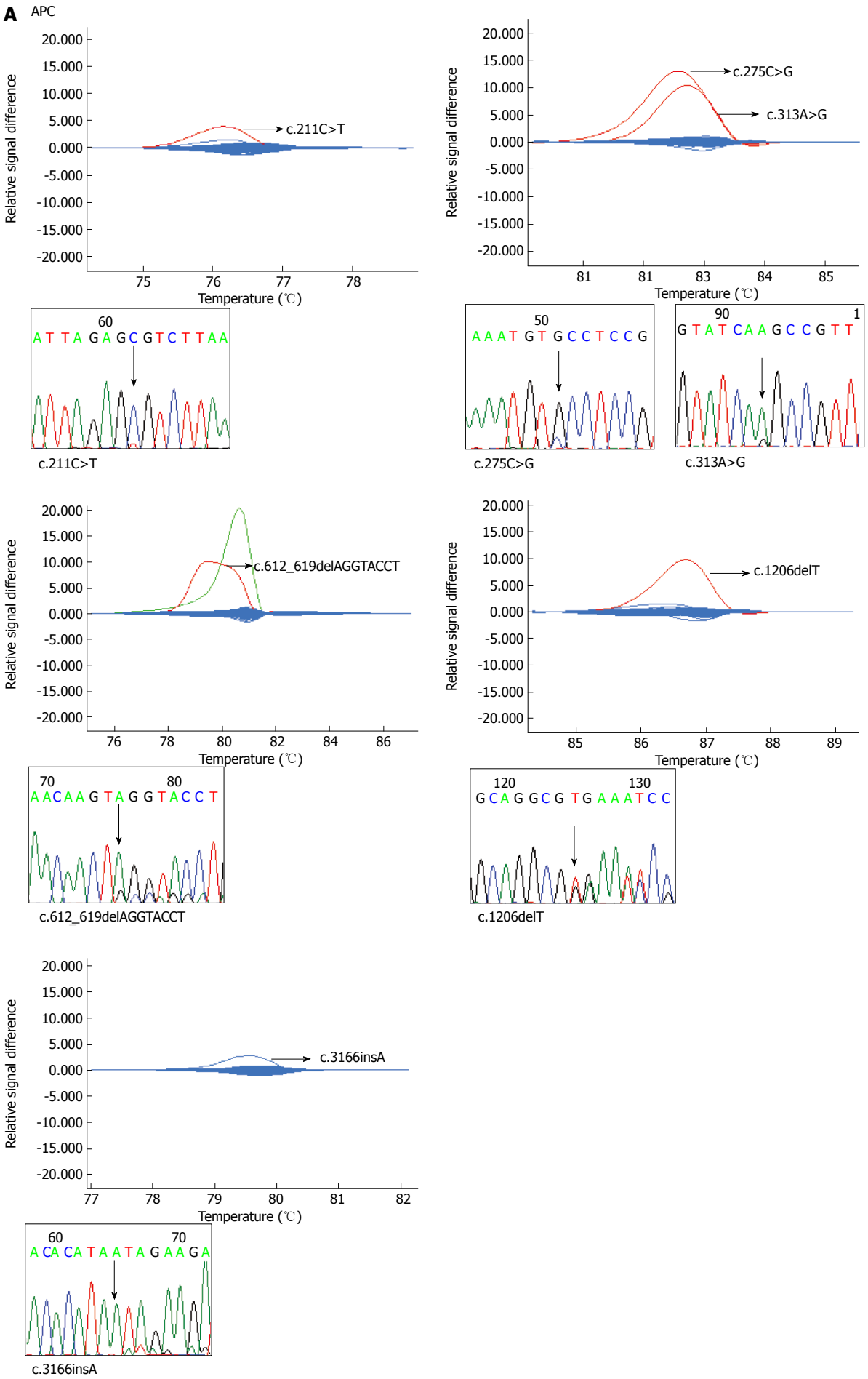
Statistical analysis

The results were analyzed using SPSS version 17.0 program. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Novel pathological mutations in the 13 driver genes

In total, we validated 18 mutations in six genes that have not been previously described in a public database (Figure 1). Six of these mutations occurred in the *APC* gene (c.211C>T, c.275C>G, c.313A>G, c.612_619delAGGTACCT, c.1206delT and c.3166insA), one in the *MLH1* gene (c.629C>T), three in the *MSH2* gene (c.166G>T, c.232G>A and c.718delG), one in the *PMS2* gene (c.1313A>C), three in the *SMAD4*


A
APC
Relative signal difference
Temperature (℃)
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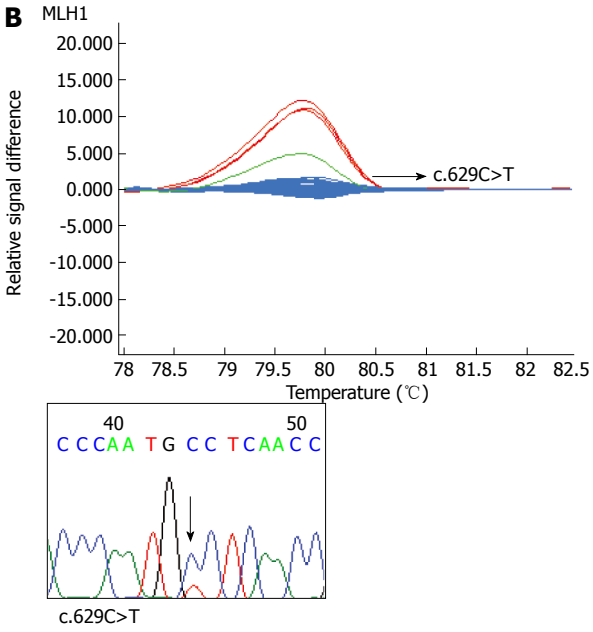
c.275C>G
c.313A>G
c.612_619delAGGTACCT
c.1206delT
c.3166insA


B
MLH1
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.629C>T
78 78.5 79 79.5 80 80.5 81 81.5 82 82.5
Temperature (℃)
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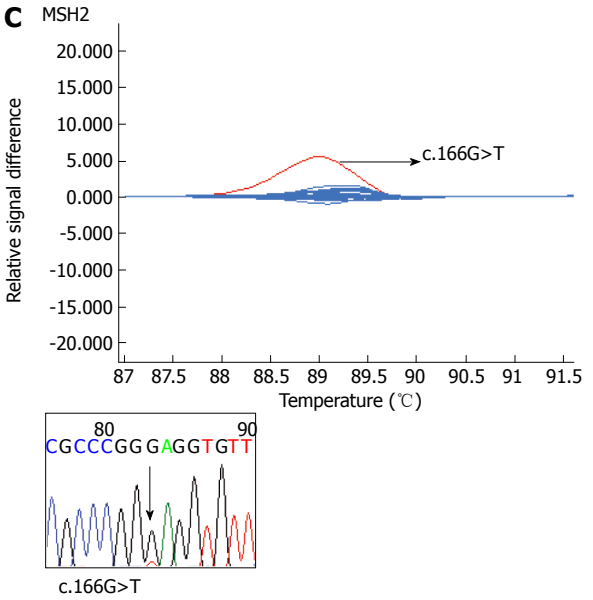
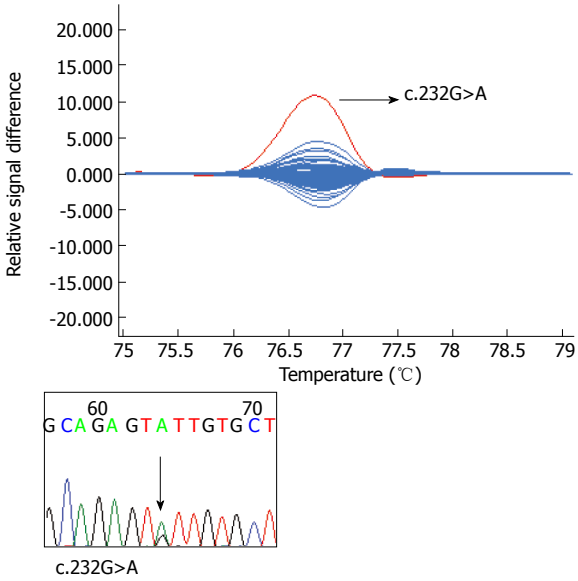
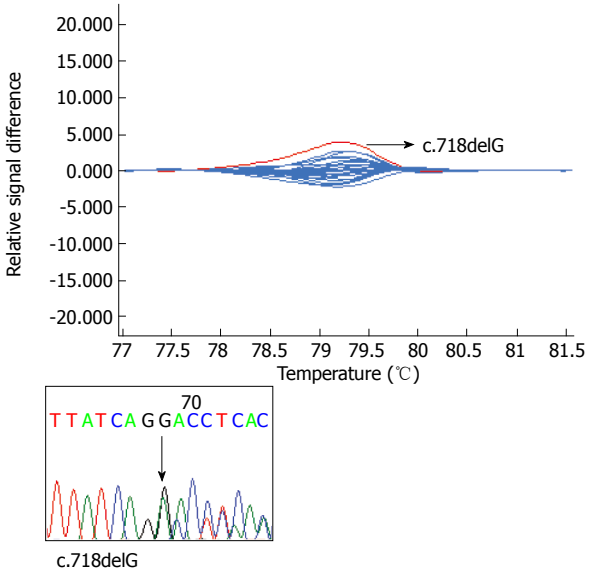
c.629C>T


C
MSH2
Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.166G>T
87 87.5 88 88.5 89 89.5 90 90.5 91 91.5
Temperature (℃)
80 90
C G C C C G G G A G G T G T T
c.166G>T


Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.232G>A
75 75.5 76 76.5 77 77.5 78 78.5 79
Temperature (℃)
60 70
G C A G A G T A T T G T G C T
c.232G>A


Relative signal difference
20.000
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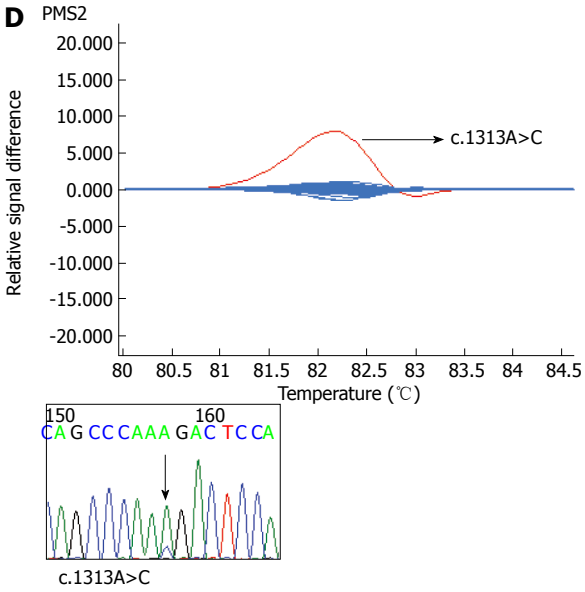
c.718delG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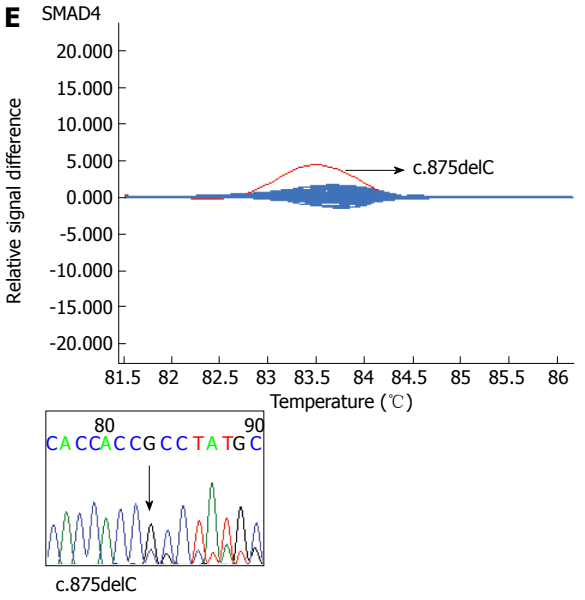
-20.000
c.1313A>C
80 80.5 81 81.5 82 82.5 83 83.5 84 84.5
Temperature (℃)
150
160
CA G CC C AAA G AC TC CA
c.1313A>C


E
SMAD4
Relative signal difference
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15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
c.875delC
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Temperature (℃)
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90
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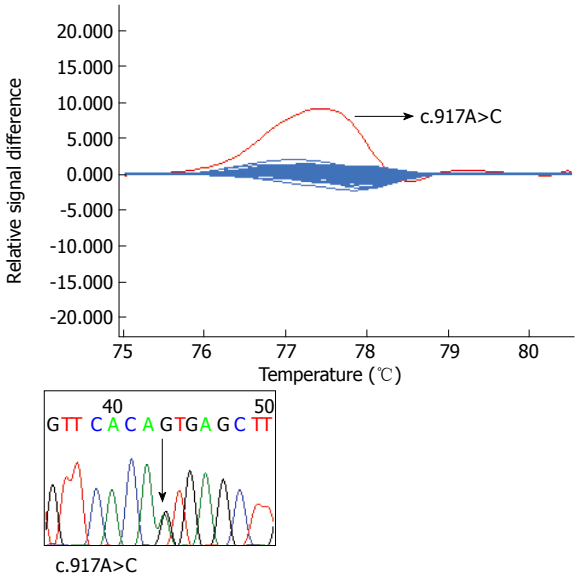
c.875delC


Relative signal difference
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15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
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50
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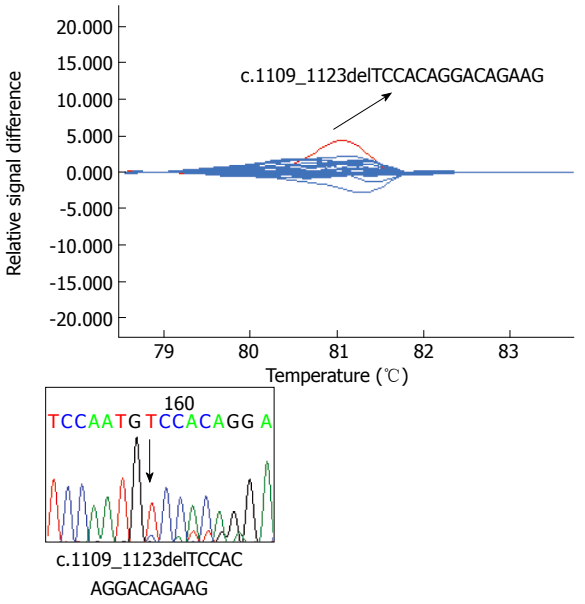
c.917A>C


Relative signal difference
20.000
15.000
10.000
5.000
0.000
-5.000
-10.000
-15.000
-20.000
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79 80 81 82 83
Temperature (℃)
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AGGACAGAAG

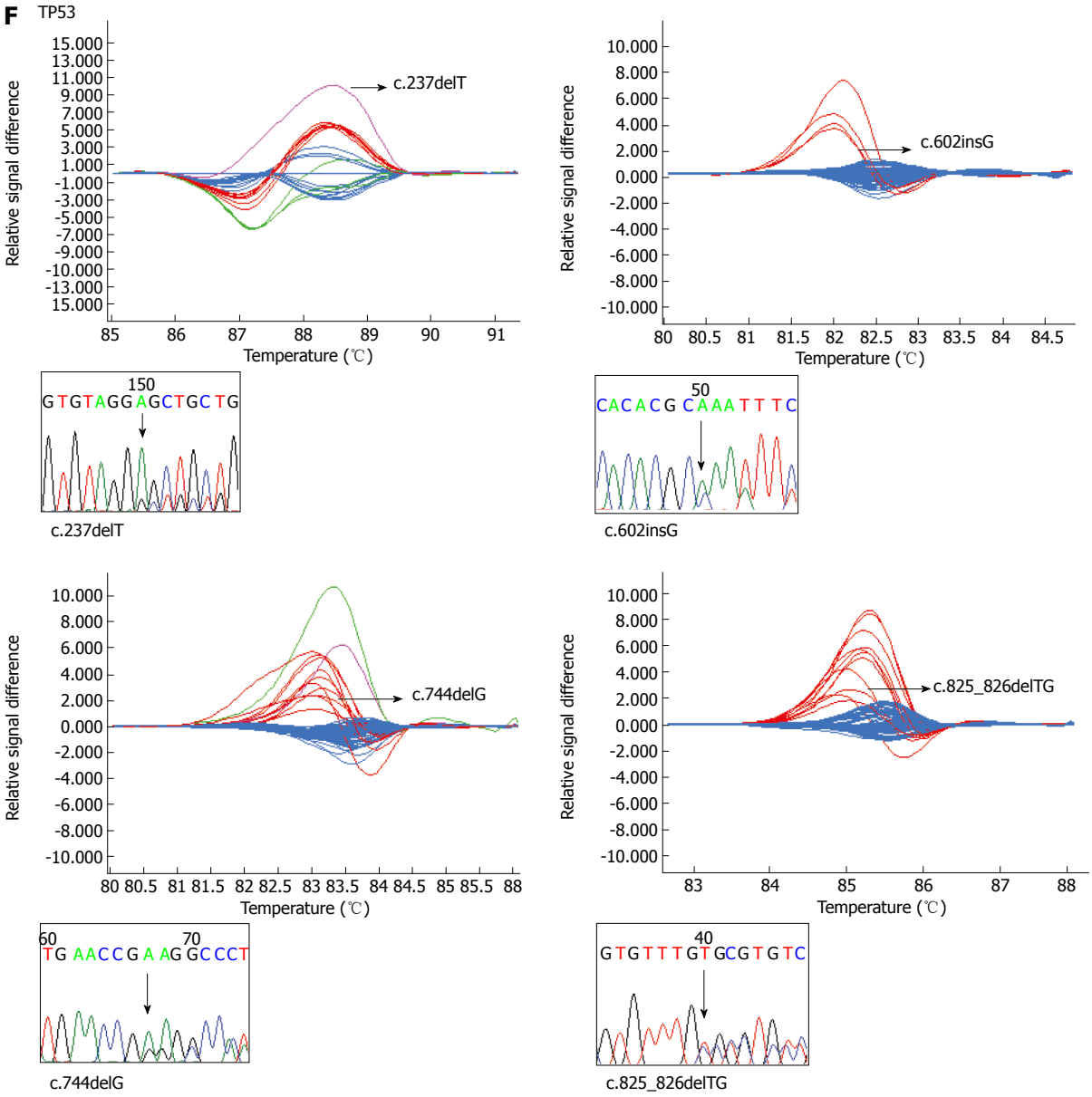

Figure 1 Direct sequencing was used to validate novel pathological mutations. A: *APC*; B: *MLH1*; C: *MSH2*; D: *PMS2*; E: *SMAD4*; F: *TP53*.

gene (c.875delC, c.917A>G and c.1109_1123delTCCACAGGACAGAAG) and four in the *TP53* gene (c.237delT, c.602insG, c.744delG and c.825_826delTG). To our knowledge, these mutations have not been detected in human cancers prior to this study.

De novo mutations in the 13 driver genes

In total, we found 16 *de novo* mutations in seven genes that have been previously described in a public database with the exception of *APC* c.465A>G, these mutations were detected in cancerous tissues only, and not in the patient's blood cells (Table 1). Three of these mutations occurred in the *APC* gene (c.465A>G, c.573T>C and c.1005A>G), one in the *BMPR1A* gene (c.1578A>G), five in the *MLH1* gene (c.462C>T, c.655A>G, c.1151T>A, c.1742C>T and c.2101C>A), two in the *MSH2* gene (c.23C>T and c.1886A>G), two in the *MSH6* gene (c.3488A>T and c.4065_4066insTTGA), two in the *MUTYH* gene (c.1422G>C and c.1440C>T) and one in the *PMS2* gene (c.1532C>T).

Polymorphisms in the 13 driver genes

In total, we validated 18 polymorphisms in six genes previously described in a public database (Table 2). One of them occurred in the *BMPR1A* gene (c.4C>A), three in the *MSH2* gene (c.471C>A, c.1168C>T and c.1690A>G), two in the *MSH6* gene (c.116G>A and c.3306T>A), one in the *MUTYH* gene (c.1014G>C), ten in the *PMS2* gene (c.59G>A, c.288C>T, c.780C>G, c.1408C>T, c.1454C>A, c.1621G>A, c.2253T>C, c.2324A>G, c.2340C>T and c.2570G>C) and one in the *TP53* gene (c.215C>G). Some of the frequencies are similar to those in a public database, but some are

Table 1 ***De novo*** **mutations of 13 driver genes detected in colorectal cancer**

Gene	Mutation	Protein change	rs number	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
APC	c.465A>G	p.Lys155=		1 (0.97)	0.49%		
	c.573T>C	p.Tyr191=	rs185154886	1 (0.97)	0.49%	NA	C = 0.06
	c.1005A>G	p.Leu335=	rs3797704	1 (0.97)	0.49%	G = 0%	G = 0.06
BMPR1A	c.1578A>G	p.Glu526=	rs202030576	1 (0.97)	0.49%	NA	G = 0.02
MLH1	c.462C>T	p.Asp154=	rs192938577	1 (0.97)	0.49%	NA	T = 0.02
	c.655A>G	p.Ile219Val	rs1799977	7 (6.8)	3.40%	G = 0%-37.5%	G = 12.96
	c.1151T>A	p.Val384Asp	rs63750447	4 (3.88)	1.94%	NA	A = 0.52
	c.1742C>T	p.Pro581Leu	rs63751684	1 (0.97)	0.49%	NA	T = 0.12
	c.2101C>A	p.Gln701Lys	rs63750114	1 (0.97)	0.49%	NA	A = 0.12
MSH2	c.23C>T	p.Thr8Met	rs17217716	3 (2.91)	1.46%	T = 0%-5%	T = 0.52
	c.1886A>G	p.Gln629Arg	rs61756468	3 (2.91)	1.46%	NA	G = 0.22
MSH6	c.3488A>T	p.Glu1163Val	rs63750252	2 (1.94)	0.97%	NA	T = 0.28
	c.4065_4066insTTGA	p.Lys1328Aspfs	rs55740729	1 (0.97)	0.49%	NA	TTGA = 0.8
MUTYH	c.1422G>C	p.Thr474=	rs74318065	1 (0.97)	0.49%	NA	G = 1.04
	c.1440C>T	p.Thr480=	rs150269172	3 (2.91)	1.46%	NA	A = 0.4
PMS2	c.1532C>T	p.Thr511Met	rs74902811	4 (3.88)	1.94%	NA	A = 3.69

Table 2 Polymorphisms of 13 driver genes detected in colorectal cancer

Gene	Mutation	Protein change	rs number	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
BMPR1A	c.4C>A	p.Pro2Thr	rs11528010	41(39.81)	19.9%	NA	A = 49.98
MSH2	c.471C>A	p.Gly157=	rs61756463	5 (4.85)	2.43%	NA	A = 0.24
	c.1168C>T	p.Leu390Phe	rs17224367	5 (4.85)	2.43%	T = 0%-4.7%	T = 0.28
	c.1690A>G	p.Thr564Ala	rs55778204	3 (2.91)	1.46%	NA	G = 0.06
MSH6	c.116G>A	p.Gly39Glu	rs1042821	1 (0.97)	0.49%	NA	A = 20.29
	c.3306T>A	p.Thr1102=	rs2020910	41 (39.81)	37.38%	A = 0%-22.9%	A = 4.93
MUTYH	c.1014G>C	p.Gln338His	rs3219489	51 (49.51)	27.67%	C = 45.2%-46.7%	C = 31.35
PMS2	c.59G>A	p.Arg20Gln	rs10254120	10 (9.71)	4.85%	NA	T = 7.57
	c.288C>T	p.Ala96=	rs12532895	58 (56.31)	33.5%	A = 28.2%-36%	A = 11.36
	c.780C>G	p.Ser260=	rs1805319	17 (16.5)	8.25%	G = 4.8%-8%	G = 16.87
	c.1408C>T	p.Pro470Ser	rs1805321	44 (42.72)	21.36%	T = 0%	T = 35.82
	c.1454C>A	p.Thr485Lys	rs1805323	44 (42.72)	21.36%	NA	T = 11.20
	c.1621G>A	p.Lys541Glu	rs2228006	15 (14.56)	7.28%	A = 4.4%-23%	A = 11.68
	c.2253T>C	p.Phe751=	rs1805325	2 (1.94)	0.97%	NA	NA
	c.2324A>G	p.Asn775Ser	rs17420802	8 (7.77)	3.88%	NA	NA
	c.2340C>T	p.Pro780=	rs142230276	8 (7.77)	3.88%	NA	A = 0.12
	c.2570G>C	p.Gly857ala	rs1802683	1 (0.97)	0.49%	NA	NA
TP53	c.215C>G	p.Pro72Arg	rs1042522	78 (75.73)	50%	G = 48.9%-61.4%	G = 45.71

not, which maybe due to ethnic differences.

Known pathological mutations in the 13 driver genes

In total, we validated 58 mutations in 10 genes that have been previously described in a public database (Table 3). Fifteen of them occurred in the *APC* gene (c.95A>G, c.646C>T, c.694C>T, c.799G>T, c.832C>T, c.904C>T, c.3907C>T, c.3914C>A, c.3914delC, c.3934G>T, c.3935delG, c.3944C>A, c.3982C>T, c.4012C>T and c.4031C>A), two in the *BRAF* gene (c.1780G>A and c.1799T>C), six in the *KRAS* gene (c.34G>C, c.34G>T, c.35G>A, c.35G>C, c.35G>T and c.38G>C), one in the *MSH2* gene (c.1480T>C), on in the *MUTYH* gene (c.74G>A), four in the *PIK3CA* gene (c.1624G>A, c.1633G>A, c.1636C>G and c.3104A>G), one in the *PMS2* gene (c.2437C>T), one in the *PTEN* gene (c.19G>T), three in the *SMAD4* gene (c.1067C>G, c.1069T>C and c.1081C>T) and 24 in the *TP53* gene (c.318C>G, c.423C>G, c.440T>G, c.511G>T, c.514G>T, c.524G>A, c.536A>G, c.586C>T, c.638G>T, c.646G>A, c.700T>G, c.734G>A, c.742C>T, c.761T>G, c.772G>A, c.772G>T, c.817C>T, c.818G>A, c.841G>C, c.844C>T, c.853G>A, c.856G>A, c.857A>G and c.1015G>T).

Distribution of mutations in CRC-related pathways

To explore the patterns of mutations in the candidate pathways, we divided the 13 driver genes into six pathways: WNT, TGF-β, PI3K, RTK-RAS, P53, and DNA repair pathways. The *TP53* gene in the P53 pathway has a relatively high rate of mutation compared with genes in the WNT, TGF-β, PI3K, RTK-RAS, and DNA repair pathways.

In total, 76 patients (73.79%) had mutations in one

Table 3 Known pathological mutations of 13 driver genes detected in colorectal cancer

Gene	Mutation	Protein change	rs number in dbSNP/mutation id in COSMIC	Number of mutations, *n* (%)	Minor allele frequency in cancer	Minor allele frequency in Asian	GMAF (global minor allele frequency%)
APC	c.95A>G	p.Asn32Ser	rs539108537	1 (0.97)	0.49%	NA	G = 0.02
	c.646C>T	p.Arg216Stop	rs62619935	1 (0.97)	0.49%	NA	NA
	c.694C>T	p.Arg232Stop	rs397515734	1 (0.97)	0.49%	NA	NA
	c.799G>T	p.Gly267Stop	The UMD-APC mutations database	1 (0.97)	0.49%	NA	NA
	c.832C>T	p.Gln278Stop	The UMD-APC mutations database	1 (0.97)	0.49%	NA	NA
	c.904C>T	p.Arg302Stop	rs137854568	1 (0.97)	0.49%	NA	NA
	c.3907C>T	p.Gln1303Stop	COSM13728	1 (0.97)	0.49%	NA	NA
	c.3914C>A	p.Ala1305Glu	COSM1432302	1 (0.97)	0.49%	NA	NA
	c.3914delC	p.Ala1305Glufs	COSM19687	1 (0.97)	0.49%	NA	NA
	c.3934G>T	p.Gly1312Stop	COSM18817	1 (0.97)	0.49%	NA	NA
	c.3935delG	p.Gly1312Glufs	COSM18796	1 (0.97)	0.49%	NA	NA
	c.3944C>A	p.Ser1315Stop	COSM18777	1 (0.97)	0.49%	NA	NA
	c.3982C>T	p.Gln1328Stop	rs398123121	3 (2.91)	1.46%	NA	NA
	c.4012C>T	p.Gln1338Stop	rs121913327	3 (2.91)	1.46%	NA	NA
	c.4031C>A	p.Ser1344Stop	COSM19135	1 (0.97)	0.49%	NA	NA
BRAF	c.1780G>A	p.Asp594Asn	rs397516896	1 (0.97)	0.49%	NA	NA
	c.1799T>C	p.Val600Glu	rs113488022	3 (2.91)	1.46%	NA	NA
KRAS	c.34G>C	p.Gly12Cys	rs121913530	2 (1.94)	0.97%	NA	NA
	c.34G>T	p.Gly12Ser	rs121913530	2 (1.94)	0.97%	NA	NA
	c.35G>A	p.Gly12Ala	rs121913529	2 (1.94)	0.97%	NA	NA
	c.35G>C	p.Gly12Asp	rs121913529	11 (10.98)	5.34%	NA	NA
	c.35G>T	p.Gly12Val	rs121913529	12 (11.65)	5.83%	NA	NA
	c.38G>C	p.Gly13Asp	rs112445441	5 (4.85)	2.43%	NA	NA
MSH2	c.1480T>C	p.Ser494Pro	rs55653533	1 (0.97)	0.49%	NA	C = 0.02
MUTYH	c.74G>A	p.Gly25Asp	rs75321043	1 (0.97)	0.49%	NA	T = 0.18
PIK3CA	c.1624G>A	p.Glu542Lys	rs121913273	1 (0.97)	0.49%	NA	NA
	c.1633G>A	p.Glu545Lys	rs104886003	1 (0.97)	0.49%	NA	NA
	c.1636C>G	p.Gln546Lys	rs121913286	1 (0.97)	0.49%	NA	NA
	c.3140A>G	p.His1047Arg	rs121913279	2 (1.94)	0.97%	NA	NA
PMS2	c.2437C>T	p.Arg813Trp	rs375968016	1 (0.97)	0.49%	NA	A = 0.02
PTEN	c.19G>T	p.Glu7Stop	COSM5298	1 (0.97)	0.49%	NA	NA
SMAD4	c.1067C>G	p.Pro356Arg	COSM339351	1 (0.97)	0.49%	NA	NA
	c.1069T>C	p.Ser357Pro	COSM189735	1 (0.97)	0.49%	NA	NA
	c.1081C>T	p.Arg361Cys	rs80338963	1 (0.97)	0.49%	NA	NA
TP53	c.318C>G	p.Ser106Arg	COSM45944	1 (0.97)	0.49%	NA	NA
	c.423C>G	p.Cys141Trp	COSM44204	1 (0.97)	0.49%	NA	NA
	c.440T>G	p.Val147Gly	COSM44309	1 (0.97)	0.49%	NA	NA
	c.511G>T	p.Glu171Stop	COSM10996	1 (0.97)	0.49%	NA	NA
	c.514G>T	p.Val172Phe	COSM44240	1 (0.97)	0.49%	NA	NA
	c.524G>A	p.Arg175His	rs28934578	5 (4.85)	2.43%	NA	NA
	c.536A>G	p.His179Arg	COSM10889	1 (0.97)	0.49%	NA	NA
	c.586C>T	p.Arg196Stop	rs397516435	2 (1.94)	0.97%	NA	NA
	c.638G>T	p.Arg213Leu	COSM43650	1 (0.97)	0.49%	NA	NA
	c.646G>A	p.Val216Met	COSM10667	1 (0.97)	0.49%	NA	NA
	c.700T>G	p.Tyr234Asp	COSM43768	1 (0.97)	0.49%	NA	NA
	c.734G>A	p.Gly245Asp	rs121912656	2 (1.94)	0.97%	NA	NA
	c.742C>T	p.Arg248Trp	rs121912651	3 (2.91)	1.46%	NA	NA
	c.761T>G	p.Ile254Ser	COSM45035	1 (0.97)	0.49%	NA	NA
	c.772G>A	p.Glu258Lys	rs121912652	1 (0.97)	0.49%	NA	NA
	c.772G>T	p.Glu258Stop	COSM43568	1 (0.97)	0.49%	NA	NA
	c.817C>T	p.Arg273Cys	rs121913343	1 (0.97)	0.49%	NA	NA
	c.818G>A	p.Arg273His	rs28934576	2 (1.94)	0.97%	NA	T = 0.02
	c.841G>C	p.Asp281His	COSM10943	1 (0.97)	0.49%	NA	NA
	c.844C>T	p.Arg282Trp	rs28934574	5 (4.85)	2.43%	NA	NA
	c.853G>A	p.Glu285Lys	rs112431538	1 (0.97)	0.49%	NA	NA
	c.856G>A	p.Glu286Lys	COSM10726	1 (0.97)	0.49%	NA	NA
	c.857A>G	p.Glu286Gly	COSM43565	1 (0.97)	0.49%	NA	NA
	c.1015G>T	p.Glu339Stop	COSM11286	1 (0.97)	0.49%	NA	NA

NA: Not available.

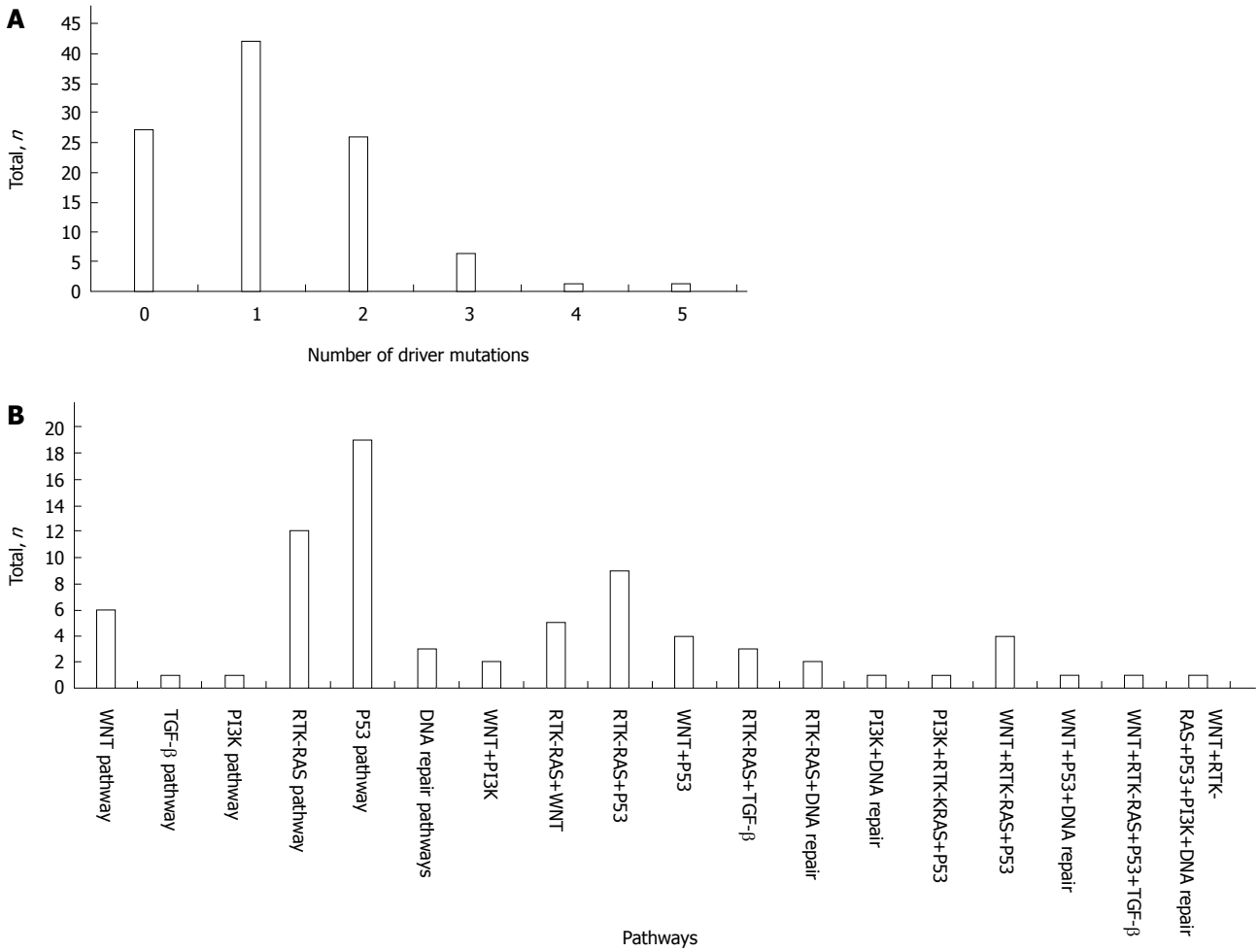

Figure 2 Mutation distribution in colorectal cancer-related pathways. A: Relationship between the number of driver mutations (horizontal axis) and number of patients (vertical axis); B: Relationship between the six major pathways (horizontal axis) and the number of patient (vertical axis).

of the 13 driver genes; of these, 42 patients (40.78%) had one driver gene mutation, and 34 patients (33.01%) had more than one driver gene mutation, including 26 patients (25.24%) with mutations in two driver genes, 6 patients (5.83%) with mutations in three driver genes, 1 patient (0.97%) with mutations in four driver genes, and 1 patient (0.97%) with mutations in five driver genes (Figure 2A).

The mutation combinations included 9 involved in the RTK-RAS/P53 pathways, 5 in the RTK-RAS/WNT pathways, 4 in the WNT/P53 pathways, 3 in the RTK-RAS/TGF-β pathways, 2 in the WNT/PI3K pathways, 2 in the RTK-RAS/DNA repair pathways, 1 in the PI3K/DNA repair pathway, 4 in the WNT/RTK-RAS/P53 pathways, 1 in the PI3K/RTK-RAS/P53 pathway, 1 in the WNT/P53/DNA repair pathway, 1 in the WNT/RTK-RAS/P53/TGF-β pathway, and 1 in the WNT/RTK-RAS/P53/PI3K/DNA repair pathway (Figure 2B).

Correlation of molecular findings with clinical data

APC mutations were significantly correlated with lymph node metastasis (P = 0.009) and cancer stage (P = 0.013) (Table 4). Other mutations did not show any significant correlation with sex, grade, lymph node involvement or stage. In addition, having mutations in two or more driver genes was correlated significantly with the degree of lymph node metastasis (P = 0.043).

DISCUSSION

The advent of next generation sequencing (NGS) has provided a powerful platform to investigate the genetic etiology of diseases. The HRM analysis is not practical for detecting mutations encompassing an entire exon or deletions of entire genes or exons; in contrast, NGS can identify the entire genetic coding sequence. NGS has been comprehensively applied in a variety of ways, including whole genome sequencing, targeted sequencing, chromatin immunoprecipitation sequencing, small RNA sequencing and transcriptome sequencing[22]. Although NGS has become the premier tool in genetic and genomic analyses, this approach can generate a large quantity of genetic data but often with high sequencing errors. Equipment, labor, reagent and supply costs for HRM analysis are significantly lower than those for NGS[23]. The HRM technique does not require complex protocols for experimental steps or data analysis, so it is faster and less expensive

Table 4 Correlation between clinicopathological features and *APC* mutation and two or more driver genes mutations

		Mutation of *APC*				Mutations of two or more driver genes			
		No	Yes	Total	*P* value	No	Yes	Total	*P* value
Gender	F	27	10	37	0.503	23	14	37	0.435
	M	52	14	66		46	20	66	
Grade	Well	4	3	7	0.443	4	3	7	0.579
	Moderate	67	19	86		57	29	86	
	Poor	8	2	10		8	2	10	
LN	-	32	17	49	0.009	28	21	49	0.043
	+	47	7	54		41	13	54	
Stage	Ⅰ, Ⅱ	30	16	46	0.013	27	19	46	0.108
	Ⅲ, Ⅳ	49	8	57		42	15	57	

P value by χ^2 test.

than NGS. We used the HRM technique to analyze the mutation profiles in CRCs of Taiwanese patients, and proved the concept.

No studies to date have assessed the frequency of non-pathological somatic mutations during oncogenesis. In our study, a *de novo* mutation was defined as a genomic alteration that was undetectable in peripheral blood and with nonpathogenic significance; however, the dbSNP database has shown the minor allele frequency for this group. Therefore, we suggest that these nucleotide changes may occur during/after cancer development, and that these mutation sites may belong to a mutational hotspot that occurs frequently in both germline and cancerous tissues.

We identified four point mutations and two deletions in the *SMAD4* gene in five CRC cases. SMAD4 plays a unique and pivotal role in the TGF-β pathway by mediating the transcriptional activation of target genes[24]. The mechanism by which mutation alters gene function is still unknown. Ling *et al*[25] proposed that a mutation in this gene may facilitate CRC progression. In our study, three samples were in the T3 stage and one was in the T4b stage, which may support this idea.

PTEN is a negative regulator of the PI3K pathway that induces cell survival and proliferation. Berg *et al*[26] found that *PTEN* mutations were more frequent in young CRC patients (< 50 years). However, in our study, we identified a *PTEN* nonsense somatic mutation in a male patient aged 66 years.

APC mutations play a critical role in CRC tumorigenesis. Some reports have indicated a potential interdependence of the two hits in *APC*, both in sporadic and FAP-associated CRCs[27,28]. In our study, one patient had two *APC* mutations outside the MCR, whereas most of patients had only one *APC* mutation. From these results, we suggest that one *APC* mutation is capable of initiating of CRC oncogenesis, similar to a *KRAS* mutation.

Tomasetti *et al*[29] showed that only three driver gene mutations are required for the development of advanced cancers in the lung and colon. In addition, they indicated that patients with MMR deficiencies that occurred through the sequential mutation of four or more driver genes significantly increased the incidence of CRC. In this study, we only analyzed 13 driver genes and were unable to confirm their findings, but we determined that 95 patients had fewer than three driver gene mutations. We suggest that further studies using NGS to sequence the exome may solve the discrepancies in these cases.

In conclusion, we identified mutations in genes such as *BRAF*, *KRAS*, *MUTYH*, *PIK3CA* and *PTEN*, as well as previously unreported point mutations or frameshift mutations in *APC*, *MLH1*, *MSH2*, *PMS2*, *SMAD4* and *TP53* genes in a group of Taiwanese CRC patients.

COMMENTS

Background

Previous genetic studies on colorectal cancer (CRC) have revealed multiple critical mutations in candidate pathways; furthermore, statistical analysis has shown that the number of driver gene mutations plays an important role in the development of CRC. However, the genetic mutations associated with CRC in the Taiwanese population are unclear.

Research frontiers

Multiple previous reports and The Cancer Genome Atlas database have revealed that several critical genes and pathways are important in the initiation, progression and treatment of CRC, these include WNT, RAS-MAPK, PI3K, TGF-β, P53, and DNA MMR pathways, and some of these mutations may affect the results of treatment.

Innovations and breakthroughs

This is the first study using high-resolution melting analysis (HRM) technique to analyze the mutation profiles in CRCs of Taiwanese patients and evaluating the frequency of non-pathological somatic mutations during oncogenesis.

Applications

The studies show that HRM analysis can be used for high-throughput mutation screening for research, as well as for molecular diagnosis and clinical purposes.

Terminology

HRM analysis is a closed-tube method, indicating that PCR amplification and subsequent analysis are sequentially performed in 1 well.

Peer-review

The manuscript entitled "Mutational analysis of 13 driver genes of colorectal cancer-related pathways in Taiwanese patients" by Chang *et al* 2015 details the use of HRM and DNA sequencing techniques applied to CRC samples, and details the identification of novel genetic mutations, as well as characterization of the prevalence of other mutations in 13 driver genes of CRC-related pathways. This paper will be of interest to scientists working in the CRC field, recommend publication.

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ORIGINAL ARTICLE

Basic Study

Choledochojejunostomy with an innovative magnetic compressive anastomosis: How to determine optimal pressure?

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Author contributions: Xue F, Liu YX and Lv Y conceived and designed the research; Xue F and Liu YX observed the magnets; Xue F, Li JP, Lu JW and Guo HC performed the research; Xue F, Li JP and Lu JW acquired the data; Xue F, Guo HC, Wang HH and Ma F performed histological studies; Xue F and Lv Y wrote the draft and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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Institutional review board statement: The entire study was carried out in strict accordance with protocols approved by the Xi'an Jiaotong University Biomedical Ethics Committee (Ethics Permit No. XJTULAC201-398).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Xi'an Jiaotong University Institutional Care and Use Committee, and performed according to the Guidelines for Animal Experimentation of Xi'an Jiaotong University (SYXK-SHAN 2014-003) and the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at luyi169@126.com. Participants gave informed consent for data sharing.

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Core tip: This study introduced a magnetic anastomosis device and verified the feasibility of magnetic compression anastomosis (magnamosis) in choledochojejunostomy; moreover, 3D printing technology was used to design and produce magnetic shells of different sizes to explore the optimum magnetic pressure range in choledochojejunostomy. The result of this study provided a more efficient and accurate theoretical basis for clinical application of choledochojejunostomy magnamosis in the future.

Xue F, Guo HC, Li JP, Lu JW, Wang HH, Ma F, Liu YX, Lv Y. Choledochojejunostomy with an innovative magnetic compressive anastomosis: How to determine optimal pressure? *World J Gastroenterol* 2016; 22(7): 2326-2335 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2326.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2326

Abstract

AIM: To investigate the optimal magnetic pressure and provide a theoretical basis for choledochojejunostomy magnetic compressive anastomosis (magnamosis).

METHODS: Four groups of neodymium-iron-boron magnets with different magnetic pressures of 0.1, 0.2, 0.3 and 0.4 MPa were used to complete the choledochojejunostomy magnamosis. Twenty-six young mongrel dogs were randomly divided into five groups: four groups with different magnetic pressures and 1 group with a hand-suture anastomosis. Serum bilirubin levels were measured in all groups before and 1 wk, 2 wk, 3 wk, 1 mo and 3 mo after surgery. Daily abdominal X-ray fluoroscopy was carried out postoperatively to detect the path and the excretion of the magnet. The animals were euthanized at 1 or 3 mo after the operation, the burst pressure was detected in each anastomosis, and the gross appearance and histology were compared according to the observation.

RESULTS: The surgical procedures were all successfully performed in animals. However, animals of group D (magnetic pressure of 0.4 MPa) all experienced complications with bile leakage (4/4), whereas half of animals in group A (magnetic pressure of 0.1 MPa) experienced complications (3/6), 1 animal in the manual group E developed anastomotic stenosis, and animals in group B and group C (magnetic pressure of 0.2 MPa and 0.3 MPa, respectively) all healed well without complications. These results also suggested that the time required to form the stoma was inversely proportional to the magnetic pressure; however, the burst pressure of group A was smaller than those of the other groups at 1 mo (187.5 ± 17.7 *vs* 290 ± 10/296.7 ± 5.7/287.5 ± 3.5, $P < 0.05$); the remaining groups did not differ significantly. A histologic examination demonstrated obvious differences between the magnamosis groups and the hand-sewn group.

CONCLUSION: We proved that the optimal range for choledochojejunostomy magnamosis is 0.2 MPa to 0.3 MPa, which will help to improve the clinical application of this technique in the future.

Key words: Choledochojejunostomy; Magnetic compressive anastomosis; Optimal range; Pressure intensity; Magnetic pressure

INTRODUCTION

Roux-en-Y choledochojejunostomy is known as a standard operation for the treatment of benign biliary stricture or malignant biliary obstruction and a well-developed approach[1]. However, the currently used manual anastomosis technique is time-consuming and associated with a high risk of complications, such as stricture recurrence and anastomotic leak[2]. Staple anastomosis has been introduced to solve this problem, but many limitations remain, such as foreign residues, low histocompatibility, and anastomosis diameter mismatch[3].

The compressive anastomosis technique was invented two centuries ago, and Denans proposed the concept of compressive anastomosis as early as 1826[4]. A new device was invented by Murphy in 1892, which has been referred to as "Murphy's button" and extensively used in intestinal anastomosis[5,6]. In 1978, Obora[7] first used magnets instead of mechanical devices; this technique cleverly avoids physical contact by using magnetic force, which is field-mediated. With advantages of simpleness, saving time, and low cost, the magnetic compressive anastomosis (magnamosis) has attracted many surgeons to solve a variety of surgical problems. Jansen *et al*[8] first successfully used magnetic rings for colorectal anastomosis in 1981. Mimuro *et al*[9] and Akita *et al*[10] reported many cases of the successful application of magnamosis for biliary strictures and biliary anastomoses in liver transplantation. In 2003, the Ventrica company launched magnetic devices used for vascular side-to-side anastomosis, and these devices were clinically successful[11,12]. Magnamosis has been proven to be a safe surgical technique that is equivalent or superior

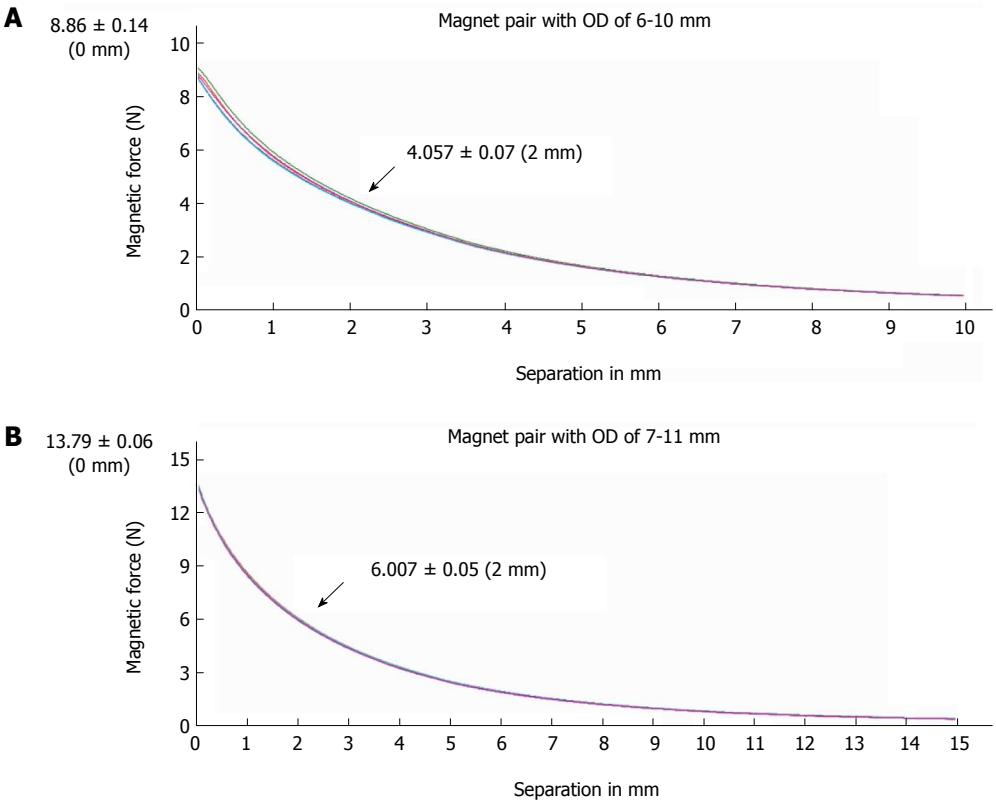


Figure 1 Magnetic force displacement curve. The magnetic force measured by a universal tensile testing machine is shown as a function of intermagnet separation (in mm) for 2 types of magnet pairs used for choledochojejunostomy magnamosis; 5 samples were tested for each magnet. A: Magnet pair with an outside diameter of 6 mm (biliary part) to 10 mm (enteric part); B: Magnet pair with an outside diameter of 7 mm (biliary part) to 11 mm (enteric part). OD: Outside diameter.

Table 1 Magnetic force of magnet pairs at the distance of 0 mm, 2 mm or 4 mm

Separation (mm)	Outside diameter of biliary - enteric magnet pairs (mm)			
	6-10	6-11	7-10	6-11
0	8.86 ± 0.14	8.24 ± 0.46	14.63 ± 0.24	13.79 ± 0.06
2	4.06 ± 0.07	4.04 ± 0.16	5.82 ± 0.09	6.01 ± 0.05
4	2.15 ± 0.03	2.30 ± 0.06	2.99 ± 0.05	3.27 ± 0.04

to anastomosis created by the hand-sewn or stapling technique[13].

Although magnetic approaches have shown promise in choledochojejunostomy, the compressive pressure and magnet specification are based on experience, and significant differences have been reported in these parameters. Furthermore, these parameters often lack systematic research, and a weaker magnetic force may create local ulceration or abscesses due to slow and contained perforation. Stronger attraction can cause severe ischemia and/or lacerating/shearing trauma, which leads to free perforation. We hypothesized that an appropriate range of magnetic pressure can create a viable and durable choledochojejunostomy, and we further proposed that the magnetic pressure affects the quality of anastomosis.

MATERIALS AND METHODS

Magnetic device preparation

The device used for the end-to-side choledochojejunostomy consisted of two parts: the biliary part and the enteric part. Both parts featured magnetic rings constructed of sintered-type neodymium-iron-boron (NdFeB, N45); the surface field was approximately 2500 GS. These magnetic materials were all plated with titanium film on the external surfaces to maintain material stability and biocompatibility. According to a previous design[14], two magnets for the biliary part were constructed with the following respective outer diameter, inner diameter and height: 6 mm × 2.5 mm × 6 mm and 7 mm × 3 mm × 6 mm. The two magnets for the enteric part were designed with the following respective outer diameter, inner diameter and height: 10 mm × 3 mm × 6 mm and 11 mm × 3 mm × 6 mm. The force-displacement curve of the biliary-enteric magnet pair with outside diameters of 6 mm-10 mm and 7 mm-11 mm were measured using a universal tensile testing machine (UTM6202, Suns Technology Stock Co., Ltd., Shenzhen, China) (Figure 1). The magnetic forces of different magnet pairs (6-10 mm, 6-11 mm, 7-10 mm, and 7-11 mm) at separation distances of 0 mm,

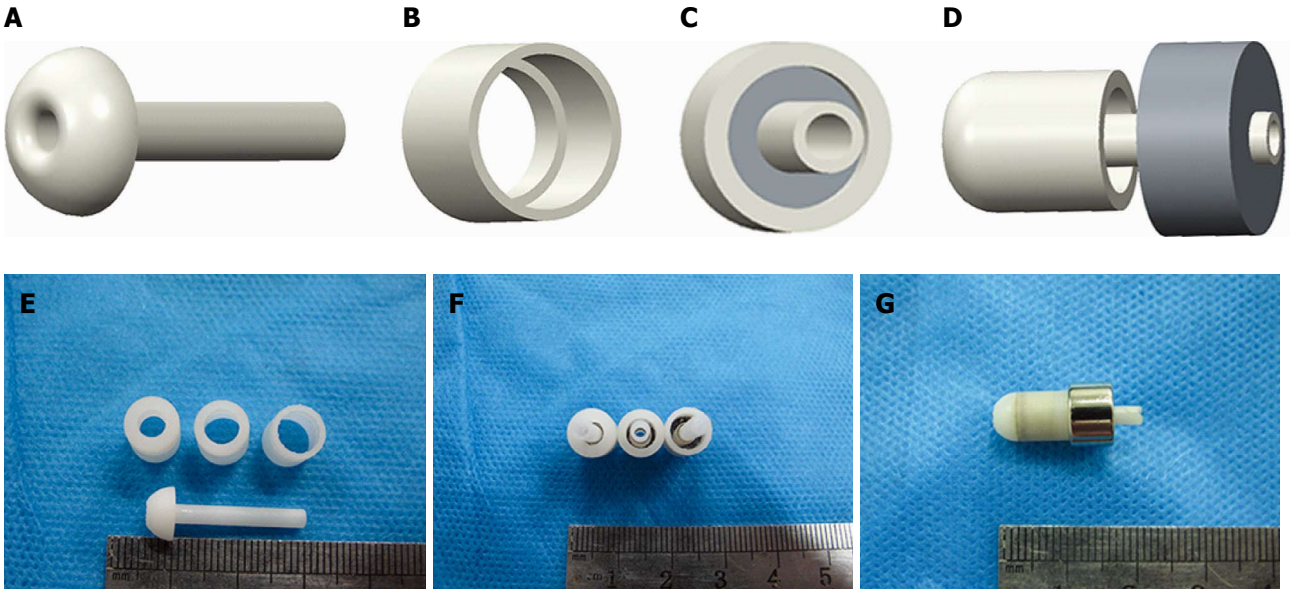


Figure 2 Mode pattern and real pictures of magnet devices. A: Lateral view of an internal drainage tube; B: View of the biliary part magnet shell; C: Antapical view of the combined biliary part; D: Lateral view of biliary part approach to enteric part; E: The internal drainage tube and shells of different crimping areas; F: Magnets with different pressures; G: Biliary part and enteric part coupled together.

2 mm and 4 mm are given in Table 1.

Three dimensional printing

To optimize the magnetic pressure for choledochojejunostomy, four different pressures were tested: 0.1 MPa, 0.2 MPa, 0.3 MPa, and 0.4 MPa (1 MPa = 1 N/mm^2). According to P = F/S, the pressure can be changed by adjusting the crimping area at a constant force. Based on these calculations, we designed a series of magnetic outer shells with different crimping areas to meet the required predetermined pressure values. The shells were made of photosensitive resin and produced using a three dimensional (3-D) printer (Figure 2). The shells feature an internal drainage duct with an inside diameter of 1.5 mm at the middle, which allowed bile to flow through and the enteric part to precisely couple with the biliary part.

Animals and grouping

Twenty-six mongrel male dogs older than 1 year and weighing more than 15 kg were provided by the Experimental Animal Center (SYXK-SHAN 2014-003) of the School of Medicine of Xi'an Jiaotong University. Male animals were selected because they cannot menstruate or become pregnant. The animals were randomly assigned to five groups: A (n = 6) - choledochojejunostomy with 0.1 MPa magnetic pressure, B (n = 6) - choledochojejunostomy with 0.2 MPa magnetic pressure, C (n = 6) - choledochojejunostomy with 0.3 MPa magnetic pressure, D (n = 4) - choledochojejunostomy with 0.4 MPa magnetic pressure, and E (n = 4) - choledochojejunostomy with traditional suture. The end-to-side enteroenteric anastomosis for the Roux-en-Y choledochojejunostomy of each group was completed using 3-0 non-absorbable sutures. One and 3 mo after surgery, postoperative complications, the bursting pressure of anastomoses, gross appearance, and pathology were evaluated.

Animal ethical approval

The experimental protocol was approved by the Animal Experimentation Committee of Xi'an Jiaotong University (No. XJTULAC201-398), and the animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. They were euthanized using a barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection, and all dogs received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Surgical procedure

First, a common bile duct dilation animal model was established in all dogs. After being fasted for 12 h and water deprived for 4 h, the dogs were anesthetized by an intraperitoneal injection of 30 g/L pentobarbital (1 mL/kg). Penicillin (2400000 U) was injected intramuscularly 30 min before surgery to avoid postoperative infection. After disinfection with povidone iodine, sterile towels were placed and an abdominal midline incision was made. By fully exposing the hepatoduodenal ligament, the common bile duct could be easily identified. 3-0 Mersilk (Ethicon; Johnson & Johnson Medical Ltd., Shanghai, China) sutures were used to ligate the distal end of the common bile duct near the duodenum (Figure 3A). Water was freely available after recovery from anesthesia, but food was

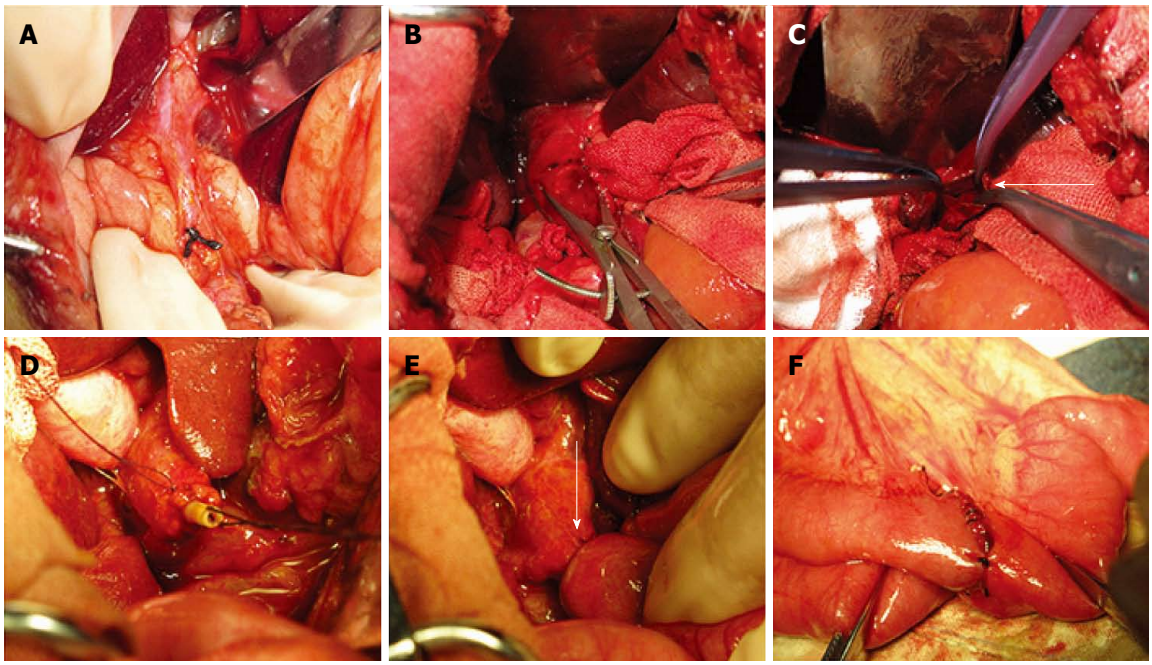


Figure 3 Surgical procedure. Images illustrating the surgical procedure with a magnet pair. A: The ligation of the distal end of common bile duct near the duodenum. B: Obvious dilatation of the common bile duct can be observed 10 d after ligation; C: Opened common bile duct before placing the biliary part magnet (arrow); D: The biliary part magnet was fixed to the stump of the common bile duct by a purse string; E: The choledochojejunostomy was constructed with the magnet pair (arrow); F: Suture enteroenteric anastomosis between the proximal end of the jejunum and the distal 50 cm of the Roux-en-Y limb.

not provided until 2 d later. Penicillin (2400000 U) was injected twice daily after surgery for 3 consecutive days.

Ten days after ligation, the dogs showed obvious symptoms of biliary obstruction such as dark-yellow urine and clay-colored stools. Serum bilirubin also significantly increased, confirming that the animal model was successfully established (Figure 3B).

The repair and reconstruction process was then performed. After the same presurgical procedures described in the common bile duct ligation operation, a second laparotomy was performed. The jejunum was dissociated and cut off approximately 15 cm away from the ligament of Treitz, and the distal end was closed with a suture. An end-to-side anastomosis was created between the proximal end and jejunum 50 cm away from the distal end with a double-layer suture of 3-0 Mersilk (Ethicon; Johnson & Johnson Medical Ltd., Shanghai, China).

In groups A, B, C and D, duct parts of the devices of different magnetic pressures were inserted into the proximal end of the common bile duct, and the stump of the bile duct was purse-string fixed with 5-0 Vicryl (Ethicon; Johnson & Johnson, Somerville, New Jersey, United States) and ligated onto the internal drainage tube. The jejunum was then punched 5 cm distal to the raised loop, and the enteric part magnet was inserted into the jejunum and coupled with the duct part magnet under the guidance of a drainage tube through the punch hole. After confirming that the common bile duct wall and the intestinal wall were clamped between the two magnets, the stump of the jejunum was then closed with 3-0 non-absorbable sutures.

Group E was subjected to a hand-sewn biliary-enteric bypass using 5-0 Vicryl, and full-thickness puncture and the mucosa-to-mucosa contact of the duct and jejunum were confirmed. The abdominal wall was then closed layer by layer (Figure 3C-E).

No food was allowed until the third day after the operation, and water was freely available. Penicillin sodium (2400000 U) was postoperatively injected intravenously twice daily for 3 d.

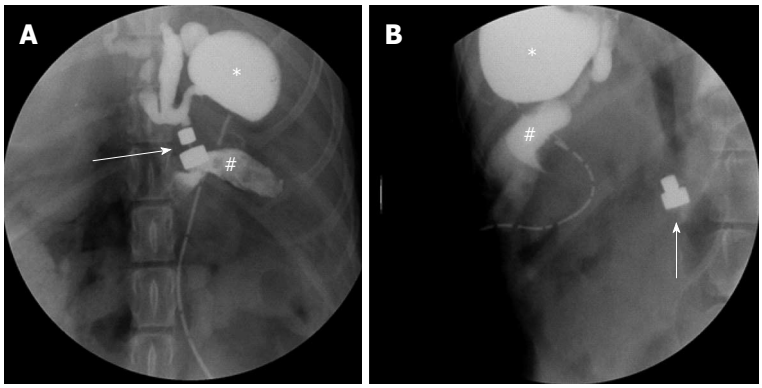
Blood test and follow-up

Blood samples for total bilirubin tests were collected at each time point, including before the ligation of the common bile duct and 10 d after ligation; 1, 2 and 3 wk after choledochojejunostomy and 1 and 3 mo after surgery.

Postoperative complications mainly included biliary stenosis and bile leakage. Biliary stenosis is reflected by recurrent jaundice and a rebound in the total bilirubin. Bile leakage can be judged by the animal state and postoperative cholangiography results. In case of death, dogs were carefully autopsied to determine the exact cause.

X-ray examination

After choledochojejunostomy, a plain abdominal X-ray was immediately taken to confirm the accurate coupling of the two magnets. The X-ray test and cholangiography were carried out every day after surgery to verify the passage of the magnets until they disappeared in the



Postoperative Postoperative 7 d

Figure 4 Postoperative abdominal X-ray. The path of the magnet pairs was monitored *via* an abdominal X-ray examination. The arrows indicates the magnetic pair, the asterisk indicates the gallbladder and the pound sign indicates the jejunum. A: Abdominal X-ray examination immediately after surgery; the two parts of the magnet pair coupled very well, exhibited good patency and were leak free; B: Daily abdominal radiography until the magnets were shed from the anastomosis.

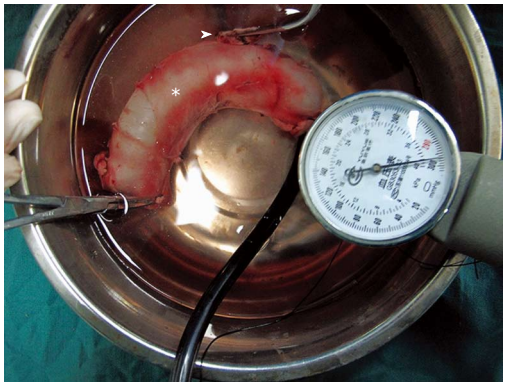


Figure 5 Bursting pressure test. Mechanical interrogation of anastomotic integrity. The arrowhead indicates the common bile duct, and the asterisk indicates the jejunum. The pointer on the sphygmomanometer is almost at the maximum, but bubbles were not observed.

photograph. The time required to shed the magnets from the anastomosis was accurately recorded for different magnet pressures (Figure 4).

Bursting strength test

The burst pressure was measured in each anastomosis. The two ends of the anastomotic specimen were ligated using hemostatic forceps or silk sutures, and the third end was attached to the sphygmomanometer and submerged in water. The intraluminal pressure was then gradually increased, and the readings were recorded as air first rose in bubbles to the surface of the water (Figure 5). The maximum hydraulic pressure when the specimen ruptured was recorded.

Tissue harvest

Dogs from each group were sacrificed at 1 and 3 mo (half of animals were sacrificed at 1 mo, and the other half were sacrificed at 3 mo), and the anastomotic specimens were harvested. After a gross observation, the specimens were cut into sections, fixed in 10% neutral formalin for subsequent mounting and stained on slides.

Statistical analysis

The statistical methods of this study were reviewed by Qian Li from the First Affiliated Hospital at Xi'an Jiaotong University.

For descriptive statistics, the data were evaluated by analysis of variance (ANOVA) and student's *t*-test. In all of the tests, the significant level was set at $P < 0.05$. The data were analyzed using the SPSS 17.0 software.

RESULTS

Total bilirubin

To accurately assess the patency of anastomosis, the initial bilirubin levels were ensured to be normal in each group. These levels significantly increased within 10 d after ligation, decreased during postoperative 1 wk and returned to normal within 1 mo. Bile leakage occurred in group A, which resulted in a faster bilirubin decrease than in the other groups (ANOVA, $P < 0.05$); in the manual group with stenosis formation, the bilirubin levels slightly increased 3 mo after a decline to normal levels (ANOVA, $P < 0.01$) (Figure 6).

Postoperative complications

Four dogs in group A experienced postoperative bile leakage, and 3 animals died of anastomosis leakage because of a failure of coupling. In addition, all dogs in group D died within one week because of severe bile leakage. The total mortality rate of 26 dogs was 27% (7/26), and the incidences of postoperative complications was 34.6% (9/26) (Table 2).

Stoma molding time

Daily abdominal radiography was performed strictly to monitor the exact time of anastomosis formation. We found that the magnet shedding time decreased as the pressure gradually increased; the mean times were

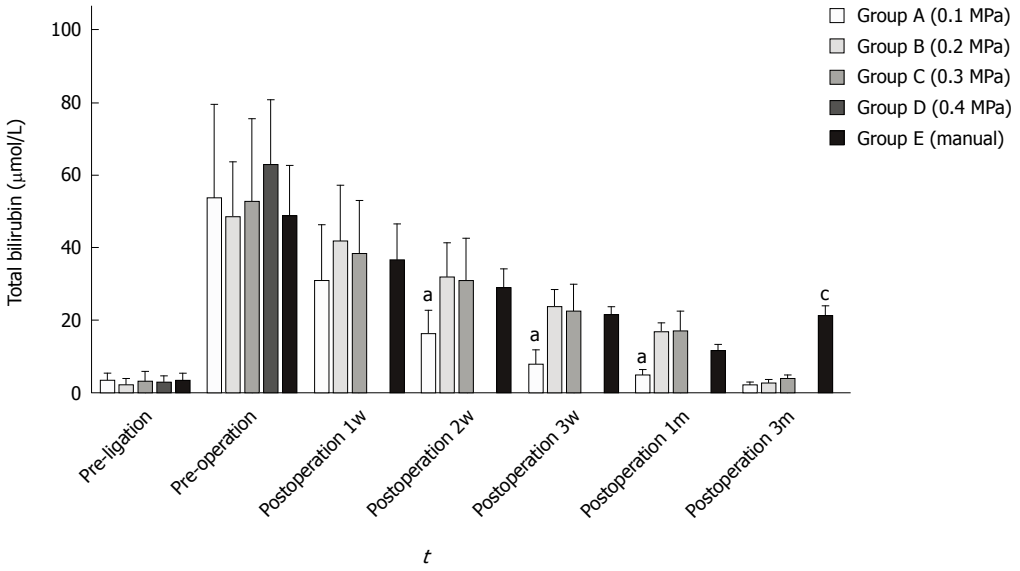
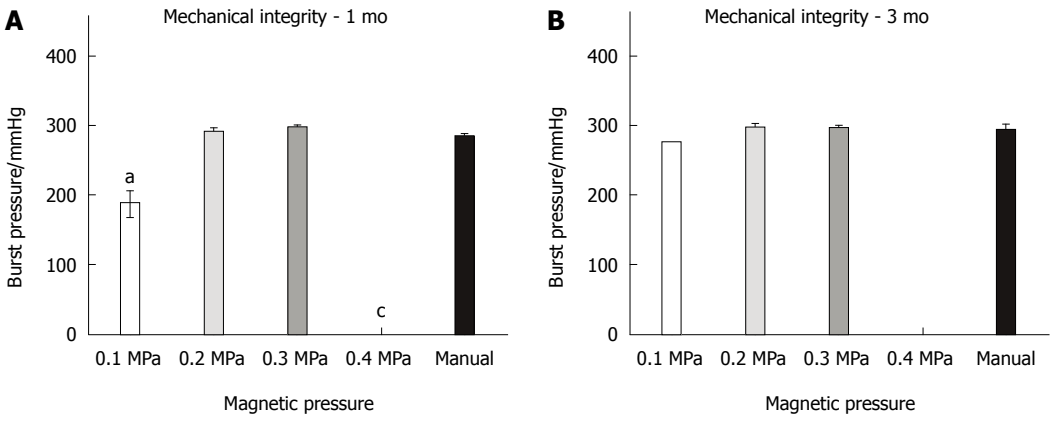


Figure 6 Serum total bilirubin between each group at different time points. Group A exhibited a faster decrease 2 wk, 3 wk and 1 mo after surgery (ANOVA, $^{a}P < 0.05$); group E exhibited a significant increase within 3 mo (ANOVA, $^{c}P < 0.05$); data were not obtained from group D due to a high mortality rate.



$^{a}P < 0.05$ for group A *vs* others

^{c}P No tissue available in group D for measurement

Figure 7 Anastomosis mechanical integrity at 1 (A) and 3 mo (B). The burst pressure at 1 mo was significantly lower in group A than in other groups (ANOVA, $P < 0.05$). The burst pressure did not differ between groups at 3 mo (ANOVA, $P = 0.052$).

9.3 ± 0.6 d for group A, 6.3 ± 0.82 d for group B and 4.5 ± 0.8 d for group D, and these times significantly differed between groups (ANOVA $P < 0.05$) (Table 2).

Bursting strength

The burst pressures at 1 and 3 mo are displayed in Figure 7. At 1 mo, the burst pressure of group A was 187.5 ± 17.7 mmHg, and this pressure significantly differed from those of groups B (290 ± 10 mmHg), C (296.7 ± 5.7 mmHg) and E (287.5 ± 3.5 mmHg) ($P < 0.05$). All dogs in group D died of complications; therefore, the exact burst pressure could not be measured. Within 3 mo, significant differences could not be observed between groups A (275 mmHg), B (296.7 ± 5.7 mmHg), C (295 ± 5 mmHg) and E (297.5 ± 3.5 mmHg) ($P = 0.052$).

Gross appearance of anastomosis

One month after the anastomosis, significant differences were observed between the traditional hand-sewn group and groups with magnamosis (Figure 8A1-E1). The suture knots remained evident in the manual group, and the mucosal surfaces appeared uneven and rough due to the interference of suture (Figure 8E1). By contrast, the mucosa of the magnamosis groups was smoother and flatter, but differences were evident between groups: an obvious anastomotic line in the mucosa can be observed in group A (Figure 8A1), whereas the mucosa was smoother in groups B and C, and the anastomosis line was not easily identified (Figure 8B1-C1). Animals in group D all experienced perforation (Figure 8D1, D2). Within 3 mo, all animals exhibited improved gross

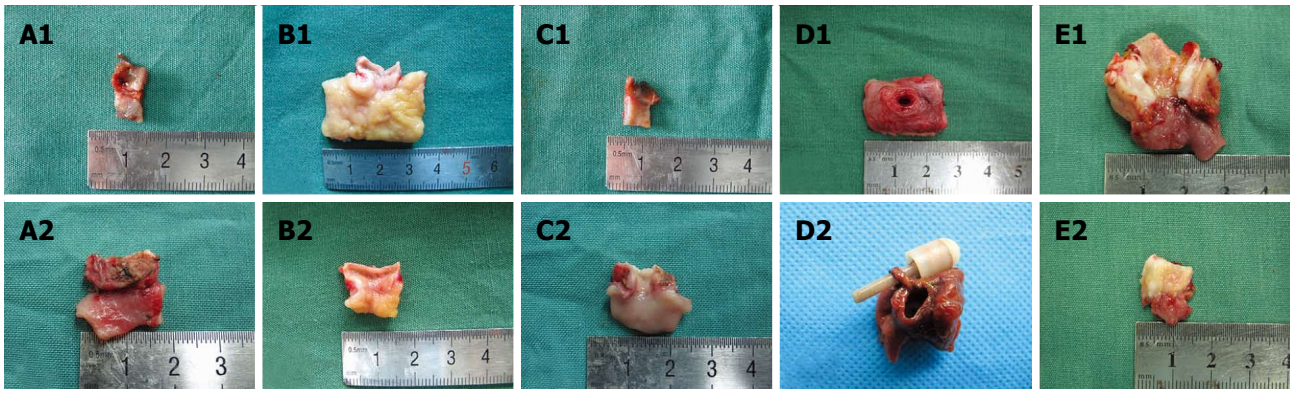


Figure 8 Gross observation of biliary-enteric anastomoses. Gross observation of anastomoses in different groups at 1 and 3 mo. A1-E1: Images of anastomoses comparing in groups A, B, C, D and E at 1 mo, respectively; A2-E2: Images of anastomoses in all groups at 3 mo.

Table 2 Postoperative outcomes of dogs and stoma molding time

Group	*n*	Complications, *n*	Death, *n*	Anastomotic molding time (d), mean ± SD
A	6	4	3	9.3 ± 0.6
B	6	0	0	6.3 ± 0.82
C	6	0	0	4.5 ± 0.84
D	4	4	4	/
E	4	1	0	/

appearances, and the inner surfaces of anastomoses healed very well (Figure 8A2-C2). Even in the manual group, almost all the suture knots disappeared from the mucosa (Figure 8E2).

Histological studies

Histological observations revealed that all groups healed very well: the submucosal layer, muscular layer and collagen fibers were appropriately organized, and a continuous epithelium migrating from the bile duct wall to the jejunum wall could be observed. Within one month, the mucous of group E sloughed and exhibited higher levels of lymphocyte infiltration compared with other groups due to foreign body stimulation (Figure 9E1). Little difference could be observed between groups A-C, and all showed mild inflammation in the submucosal region (Figure 9A1-C1). Within 3 mo, healing was completed at the anastomosis site in all groups, and the serosal, submucosal, and mucosal layers were continuous without ischemia or necrosis (Figure 9A2-E2). However, the level of lymphocytes in group E remained higher than those in the magnamosis groups (Figure 9E2).

DISCUSSION

Since the introduction of the concept of magnetic compressive anastomosis (magnamosis), it has been used to resolve certain clinical dilemmas in some case reports[15-17], including a wide range of applications in diseases of the biliary system, mainly in the treatment of biliary strictures and obstructions. Although success is not uncommon, these approaches have lacked a theoretical basis to regulate their use. Because magnamosis relies on transluminal compression, the magnetic pressure must be sufficient to effect ischemia with central necrosis such that a new channel is formed rather than an ulcer or fistula. However, the pressure must allow the surrounding non-compressed tissue to have sufficient time to remodel.

Existing research has reported that an optimized range for the bilioenteric compression force is 0.18 to 0.3 N (18-31 g) and the associated pressure can vary between 1 and 3.5 N/mm^2 (1 MPa = 1 N/mm^2)[18]. However, these data are based on the author's retrospective study of previously published studies with the help of the MAGDA online tool (http://magda.ucc.ie). Because the MAGDA tool is too idealistic to simulate the actual magnetic force and the pressure, the accuracy of this conclusion remains to be demonstrated. Thus, we adjusted the magnitude of compression and topology of the mated surfaces in this study to explore the relationship between ischemic processes and pressure and identified the most appropriate range of pressure.

Our research demonstrated the feasibility of magnamosis in performing choledochojejunostomies. The most prominent advantages of this technique are a low incidence of stenosis and the absence of postoperative residual foreign bodies. However, due to the low pressure intensity in group A, segments of tissue sandwiched between two magnets appeared viable, and the remaining ischemic and necrotic tissue eventually formed partially free perforations to cause leakage and death. Furthermore, a very long molding time was required in only three cases. In group D, excessive pressure led to larger cutting forces, which exerted the greatest effect toward the center but did not allow sufficient time for the surrounding tissue to remodel; therefore, all dogs died. In groups B and C, the moderate pressure optimized the effect of anastomosis without any postoperative complications.

To accurately monitor the anastomotic molding

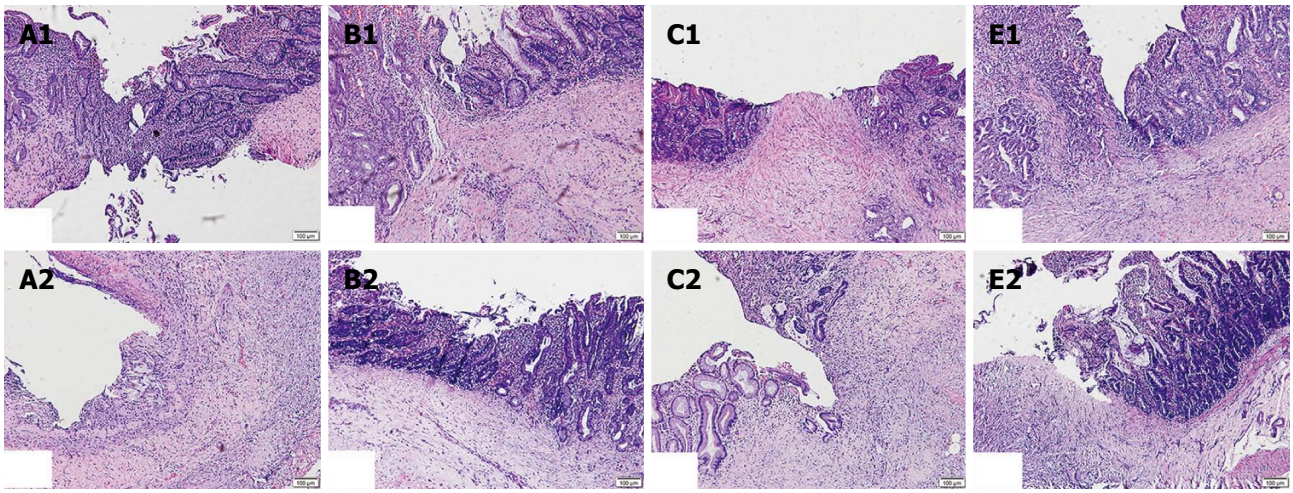


Figure 9 Histological appearance of biliary-enteric anastomoses (hematoxylin and eosin staining; original magnification × 100). Histological appearance of anastomoses in different groups at 1 and 3 mo. A1-C1 and E1: Images of anastomoses compared in groups A, B, C and E at 1 mo; A2-C2 and E2: Images of anastomoses compared in all groups at 3 mo.

time under different pressures, abdominal X-ray examinations should routinely be conducted after surgery. The results showed that the formation time was negatively correlated with the pressure - group C showed the fastest molding time; in one case, the magnets pressed on ischemic necrotic tissue and fell off on the third day. Fortunately, bile leakage was not observed under careful monitoring, the device was found in the feces two days later, and the progression of the dog was uneventful in the following days.

Although the burst pressures at 1 mo and 3 mo after surgery were similar in each group, the suture produced significant inflammation in the hand-sewn group. This inflammation easily led to fibrin deposition around the stoma and severely limited the expansion of anastomosis due to fluctuations in pressure, which increases the risk of stenosis. The burst pressure of group A was slightly lower than those of the other groups 1 mo after surgery; therefore, a lower pressure is the key factor to influence tissue anastomosis. Histopathology showed that layers of tissue did not fully heal, and obvious breakage was observed, which led to the formation of leakage. The anastomoses healed well in the other two groups with sufficient burst pressure, indicating that the magnetic pressures in these groups were ideal to complete the choledochojejunostomy magnamosis.

In conclusion, the inappropriate selection of compression characteristics may incur difficulties. This study proved that the magnetic pressure for choledochojejunostomy anastomosis can vary between 0.1 MPa to 0.3 MPa, and the optimized pressure range is 0.2 MPa to 0.3 MPa. Designing and selecting the appropriate magnet specifications will help both physicians and engineers. Further investigation remains necessary, including finite-element modeling and an analysis of optimized pressure ranges in additional, different tissues, such as gastro-enteric, entero-enteric and vascular tissues. Only such studies can provide a more rational theoretical basis for magnamosis and improve and accelerate its clinical application.

COMMENTS

Background

The magnetic compression technique (MCT), which is a simple and effective way of anastomosis, has been applied in gastroenterostomy and bilioenterostomy since it was first proposed in 1978. The authors have designed and successfully applied magnetic devices for choledochojejunostomy anastomosis. However, the blind use of these devices will result in complications. Therefore, the authors examined the effect of magnetic pressure on the effectiveness of anastomosis devices in this study. These devices were tested in animal models to determine the optimal pressure intensity in choledochojejunostomy magnamosis. The result provided a more reliable theoretical basis for the scientific and clinical application of these devices in the future.

Research frontiers

MCT utilizes a magnetic field force to achieve organ compression anastomosis, which has been widely researched and applied in bilioenterostomy and hollow organ anastomosis. However, few studies have examined the optimal magnetic pressure, and a theoretical basis is consequently lacking for these devices.

Innovations and breakthroughs

3D printing technology features advantage of high precision and easy operation. Thus, the authors combined MCT with 3D printing technology to design and produce devices used for choledochojejunostomy magnamosis with different magnetic pressures and verified the relationship between pressure gradient and tissue healing to lay a foundation for the further clinical application of these devices.

Applications

The scientific and theoretical basis provided in this study has greatly improved the safety and reliability of choledochojejunostomy magnamosis, which can be used for the treatment of obstructive jaundice as a minimally invasive approach that provides stable stomas and allows the patients to be implant free in the long term.

Terminology

MCT is a novel procedure utilizing magnetic forces for suture-less anastomoses

in hollow organs. Combined with endoscopic or interventional techniques, some conventional laparotomies may turn to be solved in a simplified minimal invasive procedure.

Peer-review

The research group performed animal experiments to examine choledochojejunostomy using MCT. Combined with 3D printing technology, the magnetic devices were cleverly designed. The animal study was well designed and executed with great innovativeness.

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ORIGINAL ARTICLE

Retrospective Study

Recurrent colorectal cancer after endoscopic resection when additional surgery was recommended

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Author contributions: Takatsu Y designed, performed research and wrote the paper; Fukunaga Y designed the research and supervised the report; Hamasaki S, Ogura A, Nagata J, Nagasaki T, Akiyoshi T, Konishi T, Fujimoto Y, Nagayama S and Ueno M supervised the report.

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Abstract

AIM: To evaluate the type of recurrence after endoscopic resection in colorectal cancer patients and whether rescue was possible by salvage operation.

METHODS: Among 4972 patients who underwent surgical resection at our institution for primary or recurrent colorectal cancers from January 2005 to February 2015, we experienced eight recurrent colorectal cancers after endoscopic resection when additional surgical resection was recommended.

RESULTS: The recurrence patterns were: intramural local recurrence (five cases), regional lymph node recurrence (three cases), and associated with simultaneous distant metastasis (three cases). Among five cases with lymphatic invasion observed histologically in endoscopic resected specimens, four cases recurred with lymph node metastasis or distant metastasis. All cases were treated laparoscopically and curative surgery was achieved in six cases. Among four cases located in the rectum, three cases achieved preservation of the anus. Postoperative complications occurred in two cases (enteritis).

CONCLUSION: For high-risk submucosal invasive colorectal cancers after endoscopic resection, additional surgical resection with lymphadenectomy is recommended, particularly in cases with lympho-

vascular invasion.

Key words: Colorectal neoplasms; Colorectal surgery; Laparoscopy; Endoscopy; Recurrence

Core tip: This is a retrospective study to evaluate the type of recurrence after endoscopic resection in colorectal cancer patients and whether rescue was possible by salvage operation. All cases were treated laparoscopically and curative surgery was achieved in six cases. Among five cases with lymphatic invasion observed histologically in endoscopic resected specimens, four cases recurred with lymph node metastasis or distant metastasis. For high-risk submucosal invasive colorectal cancers after endoscopic resection, additional surgical resection with lymphadenectomy is recommended, particularly in cases with lymphovascular invasion.

Takatsu Y, Fukunaga Y, Hamasaki S, Ogura A, Nagata J, Nagasaki T, Akiyoshi T, Konishi T, Fujimoto Y, Nagayama S, Ueno M. Recurrent colorectal cancer after endoscopic resection when additional surgery was recommended. *World J Gastroenterol* 2016; 22(7): 2336-2341 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2336.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2336

INTRODUCTION

Following recent advances in endoscopic diagnosis and techniques, the number of T1 [tumor node metastasis (TNM) cancer staging] colorectal cancer cases initially treated by endoscopic resection has been increasing[1]. Lymph node metastasis occurs in approximately 10% of cases with submucosal invasive colorectal cancers[2]. In Japan, according to the Japanese Society for Cancer of the Colon and Rectum (JSCCR) guidelines[3], when any high-risk findings are observed in histological examination of a resected specimen after endoscopic resection, additional surgical resection with lymphadenectomy is recommended. Patients with submucosal invasive colorectal cancers treated by surgical or endoscopic resection, or both, according to the JSCCR, had good clinical outcomes[2].

However, certain patients cannot undergo operation because of factors such as the patient's age and comorbid state. Particularly, in cases of rectal cancer, additional surgical resection with lymphadenectomy may cause dyschezia and a diverting stoma is sometimes necessary, which may decrease a patient's quality of life. Little is known about outcomes during follow-up in cases with endoscopic resection alone. Although some studies reporting local recurrence after endoscopic resection in T1 colorectal cancer patients for whom additional surgical resection was not indicated have been published[4-7], few reports discuss outcomes in these patients[8].

In the present study, we retrospectively reviewed eight recurrent colorectal cancer cases after endoscopic resection, for which additional surgical resection including lymphadenectomy was recommended because of high-risk submucosal invasive cancer. Our aim was to evaluate the type of recurrence and whether the patient could be rescued by a salvage operation.

MATERIALS AND METHODS

Among 4972 patients who underwent surgical resection at our institution for primary or recurrent colorectal cancers at our institution from January 2005 to February 2015, we experienced eight recurrent colorectal cancers after endoscopic resection. Additional surgical resection with lymphadenectomy had been recommended in these patients because of high-risk submucosal invasive colorectal cancer. High-risk submucosal invasive colorectal cancer after ER is defined according to the JSCCR Guidelines, when any of the following findings are observed in histological examination of the endoscopic resected specimen: (1) depth of submucosal invasion $\geqslant$ 1000 μm; (2) lymphovascular invasion was positive; (3) signet-ring cell carcinoma, mucinous carcinoma, or poorly differentiated adenocarcinoma; (4) Grade 2/3 budding; and (5) vertical tumor margin was positive. All cases had undergone endoscopic resection at another hospital and were referred to our institution after cancer recurred. The study was approved by our local institutional review board and signed consent was obtained from all patients to use data from their medical records in the study.

We retrospectively reviewed the patient characteristics including age, sex, American Society of Anesthesiologists physical status, body mass index, tumor location, and pathological data. Pathological data in six of eight cases were re-examined in our institution. All specimens from endoscopic resection were examined microscopically for resection margin status and tumor characteristics as identified above. Treatment and analysis information for recurrence after ER included the use of adjuvant therapy, follow-up interval, recurrence pattern, time to recurrence, and treatment to recurrence. Surgical outcome information in our institution included the surgical procedures, harvested lymph nodes, estimated blood loss, operating time, and complications. Postoperative outcome data included pathological findings, therapy after surgery, and survival outcome.

RESULTS

Patients' demographic data, including the primary cancer, are shown in Table 1. The median age was 60 years (range, 39-76 years) and the proportion of male patients was higher than female (six *vs* two cases, respectively). The median body mass index of all

Table 1 Characteristics of patients and primary tumors

Case	Age (yr)	Sex	ASA	BMI (kg/m^2)	Location	Pathological data					
						Invasion depth	Differentiation	ly	v	VM	Budding
1	39	F	1	19.7	Rectum	sm (≥ 1000 μm)	Well	+	+	-	Grade 2
2	43	M	1	19.4	Rectum	sm (≥ 1000 μm)	Well	+	+	+	Unknown
3	71	F	1	17.0	Rectum	sm (< 1000 μm)	Well	+	+	-	Unknown
4	75	M	1	25.3	Transverse	sm (= 1000 μm)	Well	-	-	-	Grade 0
5	76	M	2	21.7	Rectum	un clear	Moderate	-	-	+	Grade 0
6	42	M	1	18.8	Rectum	sm (≥ 1000 μm)	Well	+	+	-	Grade 0
7	74	M	2	25.4	Rectum	sm (≥ 1000 μm)	Moderate	-	+	-	Grade 3
8	49	M	2	22.4	Sigmoid	sm (≥ 1000 μm)	Moderate	+	-	-	Grade 1

Table 2 Follow up and recurrence after endoscopic resection

Case	Use of adjuvant therapy			
	After ESD	Follow up interval	Recurrence pattern	Time to recurrence
1	-	3 mo	Distant metastasis (lung, PALN)	8 mo
2	-	Every year	Local + regional lymphnode	10 mo
3	-	No follow	Distant metastasis (lung, liver)	7 yr
4	-	8 mo	Local	4 yr
5	-	No follow	Local + regional lymphnode	18 mo
6	XELOX 6 mo	No follow	Local	2 yr
7	-	Every year	Local + regional lymphnode	4 yr
8	-	No follow	Peritoneal dissemination	4 yr

Local: Intra mural; PALN: Para aortic lymphnode.

patients was 20.7 (range, 17.0-25.4) and the primary cancer was located in the rectum in six patients. Pathological examination in the previous hospitals showed that all cases indicated a need for additional surgical resection including lymphadenectomy, with the following findings: deep submucosal invasion (6 cases), lymphatic invasion positive (five cases), vascular invasion positive (five cases), vertical margin positive (2 cases), poor differentiation (0 case), and grade 2/3 budding (2 cases).

Follow-up and recurrence after endoscopic resection

Adjuvant chemotherapy after ER was performed in only one case. Four patients were followed up once every 3-12 mo after ER but another four cases were not followed up. The recurrence patterns were intramural local recurrence in five cases, regional lymph node recurrence in three cases, and associated with simultaneous distant metastasis in three cases (Table 2). Among five cases with lymphatic invasion observed histologically in the ER specimen, four cases recurred with lymph node metastasis or distant metastasis, and one case receiving adjuvant chemotherapy after ER experienced only local recurrence. Surgical outcomes are shown in Table 3. All cases were treated laparoscopically, curative surgery was achieved in six cases, and there was no conversion to open surgery. The median operating time was 284 min (range, 205-440 min) and the median estimated blood loss was 168 mL (range, 10-285 mL). Among four cases located in the rectum, three cases achieved preservation of the anus. Postoperative complications occurred in two cases (enteritis).

Among three cases with distant metastasis, laparoscopic sigmoidectomy and excision of peritoneal dissemination were performed in one case. The remaining two cases received systemic chemotherapy after staging laparoscopy or laparoscopic sigmoid colostomy.

Postoperative outcomes are shown in Table 4. Macroscopically complete resection was achieved in five cases and no recurrence occurred during the 3-106-mo follow-up. Adjuvant chemotherapy was performed in three patients who had lymph node metastasis.

DISCUSSION

Of the eight cases in our study, three experienced recurrence with distant metastasis. Follow-up was not performed for all of the patients for whom additional resection after endoscopic treatment was recommended and some cases presented with distant metastasis at the time of recurrence. Yoshii *et al*[8] compared outcomes between patients who underwent additional surgery (ER + SURG) and those who did not (ER only) and reported that the cumulative risks of recurrence was 3.7% (5/180) in the ER + SURG group *vs* 20.1% (13/96) in the ER only group (P = 0.001). The authors also emphasized 6 of 13 recurrent cases in the ER only group were associated with concurrent distant metastasis. These findings support the JSCCR guidelines that additional surgery should be performed. However, additional surgical resection with lymphadenectomy in cases of rectal cancers can be associated with permanent stoma or some degree of anal dysfunction. Certain risks of surgical complications related to the operation have also been reported. Because of these concerns as well as patient age and comorbid state, some cases were followed up by ER

Table 3 Surgical outcomes

Case	Surgical procedures	Operating time (min)	Estimated blood loss (mL)	Harvested lymph nodes	Lymphadenectomy	Complication
1	Staging laparoscopy	139	10	-	-	-
2	Laparoscopic LAR	205	10	15	D3	Enteritis
3	Laparoscopic Sigmoid colostomy	-	-	-	-	-
4	Laparoscopic TCR	339	135	12	D2	-
5	Laparoscopic LAR	440	285	10	D3	Enteritis
6	Laparoscopic APR	403	200	18	D3	-
7	Laparoscopic LAR	227	20	13	D3	-
8	Laparoscopic SCR+ Excision of peritoneal dissemination	230	350	-	-	-

LAR: Low anterior resection; TCR: Transverse colon resection; APR: Abdominoperineal resection; SCR: Sigmoid colon resection.

Table 4 Postoperative outcomes

Case	Pathological data								Survival outcome		Last follow-up
	Invasion depth	ly	v	ew	ow	aw	Lymphnode metastasis, *n*	Surgical margins	Therapy after surgery	Recurrence	(alive/death)	(mo)
1								R2	Systemic chemotherapy		Death	25
2	a	2	0	-	-	-	2	R0	Follow-up	-	Alive	106
3								R2	Systemic chemotherapy		Death	12
4	sm (700 μm)	0	0	-	-	-	0	R0	Follow-up	-	Alive	24
5	a	1	1	+	-	-	1	R1	Adjuvant chemotherapy	-	Alive	3
6	a	1	1	-	-	-	0	R0	Follow-up	-	Alive	7
7	a	0	1	-	-	-	1	R0	Adjuvant chemotherapy	-	Alive	23
8	Adenocarcinoma (recurrence of sigmoid colon cancer)							R0	Follow-up	-	Alive	33

without additional surgical resection.

Oka *et al*[9] reported that the incidence of lymph node metastasis was only 2.2%, regardless of the degree of submucosal invasion depth. Other studies also reported that patients with deep submucosal invasion only had a low cumulative risk of recurrence even without surgery[8,10,11]. Similarly, in our series, one case with deep submucosal invasion only, that experienced intramural local recurrence after ER, underwent curative surgery. Therefore, patients with only the single risk factor of deep submucosal invasion could be rescued by salvage if they are followed up.

In contrast, some studies have reported that lymphatic invasion was an independent risk factor for lymph node metastasis[2] and that venous invasion and lymph node metastasis were independent factors for a poor prognosis[2,12]. In our series, all cases with lymphatic invasion in the ER specimen recurred with distant metastasis. In patients with lymphovascular invasion, additional surgical resection is strongly recommended.

All of our cases were treated laparoscopically. Similar to findings in previous randomized clinical trials[13-15], laparoscopic surgery had less blood loss and associated with shorter hospital stay, and earlier recovery of bowel function compared with the open surgery. These results indicate that laparoscopic surgery is a feasible procedure with short-term benefits compared with open surgery.

Regarding adjuvant therapy after ER, our patient with lymphovascular invasion experienced only local recurrence after adjuvant chemotherapy. Studies of additional alternative therapy in rectal cancers have reported the effectiveness of chemoradiotherapy for patients with high-risk submucosal invasive colorectal cancers who declined additional surgery[16,17].

Surveillance after endoscopic resection is important. Some studies have reported recurrence within three to five years after curative ER[18-21]; however, the ideal follow-up period after ER with indication for additional surgery has not been determined. Yoshii *et al*[8] suggested a recommended follow-up period of at least 5 years based on the finding that their 13 cases of recurrence after ER occurred over 69 mo. Because the longest interval to recurrence was seven years in our series, it is difficult to recommend an ideal follow-up period.

In our study, two of three cases with simultaneous distant metastasis were not followed up using any modality. If ER is performed without additional surgery, we recommend monitoring closely for recurrence. Because the shortest interval to recurrence was 8 mo in our study, we recommend a follow-up interval of at least every 6 mo.

In conclusion, for high-risk submucosal invasive colorectal cancers after ER, we recommend additional surgical resection with lymphadenectomy particularly in cases with lymphovascular invasion. Patients with

high-risk submucosal invasive colorectal cancers should be adequately advised of the outcome of recurrence.

COMMENTS

Background

In Japan, according to the Japanese Society for Cancer of the Colon and Rectum guidelines, when any high-risk findings are observed in histological examination of a resected specimen after endoscopic resection, additional surgical resection with lymph node dissection is recommended. However, some patients refuse the operation because of the patient's will and comorbid state. Particularly, in cases of rectal cancer, additional surgical resection including lymph node dissection may cause dyschezia and a diverting stoma resulting decrease of patient's quality of life is sometimes necessary. Little is known about outcomes during follow-up in cases with endoscopic resection alone. Thus, it is important to evaluate the type of recurrence of these patients and whether they could be rescued by a salvage operation.

Research frontiers

Although some studies reporting local recurrence after endoscopic resection in T1 colorectal cancer patients for whom additional surgical resection was not indicated have been published, few reports discuss outcomes in the patients with endoscopic resection alone. The results of this study was persuasive and helpful for clinical.

Innovations and breakthroughs

In this study, all cases were treated laparoscopically and curative surgery was achieved in six cases. Among five cases with lymphatic invasion observed histologically in endoscopic resected specimens, four cases recurred with lymph node metastasis or distant metastasis. For high-risk submucosal invasive colorectal cancers after endoscopic resection, additional surgical resection with lymphadenectomy is recommended, particularly in cases with lymphovascular invasion.

Applications

This study suggests that for high-risk submucosal invasive colorectal cancers after endoscopic resection, additional surgical resection with lymphadenectomy is recommended, particularly in cases with lymphovascular invasion. Due to lack of cases only by this retrospective study, another case series of prospective study design or randomized control study with multicenter cooperation for correcting data will be required.

Peer-review

The authors evaluated the type of cancer recurrence after endoscopic resection in 8 colorectal cancer (CRC) patients and whether rescue was possible by salvage operation. The reviewer absolutely agrees with the main conclusion of the authors, namely for high-risk submucosal invasive CRCs additional surgical resection with lymphadenectomy is highly recommended after EMR/EMD, particularly in cases with lymphovascular invasion. Though the number of cases is small, that was persuasive and helpful for clinical.

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ORIGINAL ARTICLE

Retrospective Study

Risk factors of biliary intervention by imaging after living donor liver transplantation

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Abstract

AIM: To determine the risk factors of biliary intervention using magnetic resonance cholangiopancreatography (MRCP) after living donor liver transplantation (LDLT).

METHODS: We retrospectively enrolled 196 patients who underwent right lobe LDLT between 2006 and 2010 at a single liver transplantation center. Direct duct-to-duct biliary anastomosis was performed in all 196 patients. MRCP images routinely taken 1 mo after LDLT were analyzed to identify risk factors for biliary intervention during follow-up, such as retrograde cholangiopancreatography or percutaneous transhepatic biliary drainage. Two experienced radiologists evaluated the MRCP findings, including the anastomosis site angle on three-dimensional images, the length of the filling defect on maximum intensity projection, bile duct dilatation, biliary stricture, and leakage.

RESULTS: Eighty-nine patients underwent biliary intervention during follow-up. The anastomosis site angle [hazard ratio (HR) = 0.48; 95% confidence interval (CI), 0.30-0.75, $P < 0.001$], a filling defect in the anastomosis site (HR = 2.18, 95%CI: 1.41-3.38,

P = 0.001), and biliary leakage (HR = 2.52, 95%CI: 1.02-6.20, P = 0.048) on MRCP were identified in the multivariate analysis as significant risk factors for biliary intervention during follow-up. Moreover, a narrower anastomosis site angle (*i.e.*, below the median angle of 113.3°) was associated with earlier biliary intervention (38.5 ± 4.2 mo *vs* 62. 1 ± 4.1 mo, P < 0.001). Kaplan-Meier analysis comparing biliary intervention-free survival according to the anastomosis site angle revealed that lower survival was associated with a narrower anastomosis site angle (36.3% *vs* 62.0%, P < 0.001).

CONCLUSION: The biliary anastomosis site angle in MRCP after LDLT may be associated with the need for biliary intervention.

Key words: Magnetic resonance cholangiopancreatography; Liver transplantation; Living donor; Biliary intervention; Endoscopic retrograde cholangiopancreatography; Percutaneous transhepatic biliary drainage

Core tip: Biliary complications and interventions are common after living donor liver transplantation (LDLT). Identifying patients who are at high risk for biliary interventions early after LDLT could help clinicians with patient follow-up. Magnetic resonance cholangiopancreatography (MRCP) imaging was performed 1 mo after LDLT to determine risk factors for biliary intervention. The anastomosis site angle, a filling defect in the anastomosis site, and biliary leakage on MRCP were identified as significant risk factors. Moreover, a narrower anastomosis site angle was related to earlier biliary intervention. Here, for the first time, we have shown that the anastomosis site angle might be associated with the need for biliary intervention.

Lee SK, Choi JY, Yeo DM, Lee YJ, Yoon SK, Bae SH, Jang JW, Kim HY, Kim DG, You YK. Risk factors of biliary intervention by imaging after living donor liver transplantation. *World J Gastroenterol* 2016; 22(7): 2342-2348 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2342.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2342

INTRODUCTION

Biliary complications occur commonly after liver transplantation (LT)[1] and are the main cause of morbidity and mortality in LT recipients[2]. Magnetic resonance cholangiopancreatography (MRCP), a non-invasive tool used to visualize the biliary tract, has 88% sensitivity and 94% specificity for detecting biliary complications following LT[3]. Therefore, MRCP is the recommended diagnostic modality for detecting biliary complications after LT[3]. Biliary interventions, such as endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic biliary drainage (PTBD) are generally used to manage these complications[4].

Several investigators have documented risk factors influencing the development of biliary complications. Perioperative risk factors include a Model for End-stage Liver Disease (MELD) score > 35, donor age > 60 years, and primary sclerosing cholangitis[5,6]. Intraoperative risk factors include cold ischemic time and anastomosis method (duct-to-duct *vs* hepaticojejunal methods)[5,6]. Cytomegalovirus infection and hepatic artery thrombosis have been reported as postoperative risk factors[5,6].

However, no study has investigated the risk factors associated with the future need for intervention based on MRCP findings shortly after living donor LT (LDLT). Identifying patients at high risk for biliary intervention by MRCP, a non-invasive and accurate tool, during the early post-transplant period would be clinically helpful for managing and following patients who have undergone LT.

The purpose of this study was to determine factors, specifically the anastomosis site angle, that increase the requirement for biliary intervention during follow-up in LDLT recipients on MRCP images 1 mo after LT.

MATERIALS AND METHODS

Study population

We retrospectively reviewed the records of 270 patients who underwent LDLT at Seoul St. Mary's Hospital between 2006 and 2010. Of these 270 patients, 74 patients were excluded for the following reasons: two subjects underwent ERCP or PTBD within 1 mo after LDLT, 38 had no MRCP images taken 1 mo after LDLT, 13 had no three-dimensional (3D) reconstruction or maximum intensity projection (MIP) images or had poor quality images with severe artefacts, 3 underwent choledochojejunostomy as the biliary anastomosis method, and 18 died < 1 mo after LDLT (bleeding, 4; sepsis, 11; graft failure, 3). Finally, 196 consecutive LDLT recipients who underwent MRCP 1 mo after LT were included in this study. The present study was approved by the Institutional Review Board of Seoul St. Mary's Hospital (KC13RISI0788).

Type of graft liver and anastomosis method

All 196 patients underwent right lobe living donor transplantation[7]. Biliary anastomosis was performed according to the anatomy of the hepatic duct: single duct-to-duct anastomosis for a single hepatic duct and double duct-to-duct anastomosis, or hepaticoplasty, for double hepatic ducts. Hepaticoplasty for double hepatic ducts was performed to create one lumen in cases of a short distance between the two hepatic

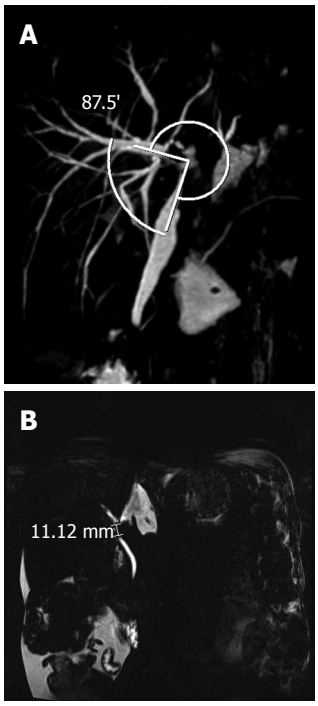

Figure 1 Representative magnetic resonance cholangiopancreatographic image showing the anastomosis site angle (A) and the filling defect (B).

ducts[8]. Alternatively, double duct-to-duct anastomosis was performed such that each duct was anastomosed to the left and right hepatic ducts individually.

Timing of biliary intervention during follow-up

Biliary intervention was defined as procedures involving ERCP and PTBD. During follow-up, these procedures were performed when the following criteria were met: (1) abnormal laboratory findings of biliary-associated factors, such as serum bilirubin, alkaline phosphatase and γ-glutamyl transferase; and (2) radiologically determined bile duct stricture with dilatation above the stricture site[9].

MRCP image analysis

MRCP was performed 1 mo after LDLT. The anastomosis site angle was measured using 3D reconstruction imaging. The anastomosis site angle was defined as the angle between the donor hepatic duct and the recipient's common hepatic duct (Figure 1A). If there were two anastomosis sites, the smaller one was chosen. To improve the accuracy of measurement of the anastomosis site, we checked the measurements on every 3D image rendered and chose the smallest site. We also identified the presence of a filling defect and the length of the filling defect on MIP images. Every image was reviewed, and the filling defect with the longest length was chosen (Figure 1B). The presence of bile duct dilatation, biliary stricture, and leakage was also verified. MRCP images were reviewed by two experienced radiologists (Lee YJ and Yeo DM) without knowledge of the patient's clinical status, and the measurements were made in consensus.

Statistical analysis

The patient characteristics are expressed as the means ± SD (range) or counts, as appropriate. The inter-observer correlation coefficient (ICC) was determined to evaluate agreement between the two radiologists for the anastomosis site angle and the length of the filling defects. The Cox proportional hazards model was used to determine risk factors for biliary intervention, such as ERCP or PTBD, after LDLT. Variables with P values < 0.2 in the univariate Cox regression analysis were considered potential variables for the multivariate Cox regression analysis. A forward selection method was adopted to identify significant risk factors with P values < 0.05. Kaplan-Meier analysis was also performed to estimate biliary intervention-free survival according to the anastomosis site angle. All statistical analyses were performed using SPSS ver. 15.0 (SPSS, Chicago, IL, United States).

RESULTS

Patient characteristics

Of the 196 patients, 136 (70.0%) were men, and the mean recipient age was 49.7 ± 10.1 years. The underlying causes for LT were hepatocellular carcinoma (n = 80, 40.8%), decompensated liver cirrhosis (LC) associated with hepatitis B (n = 51, 26.0%), alcoholic LC (n = 27, 13.8%), and hepatitis C-associated LC (n = 4, 2.0%). Eighty-nine patients (45.4%) underwent biliary intervention (Table 1). At the time of biliary intervention, jaundice (80.5%) and itching sensation (33.3%) were the main manifestations. In laboratory findings, mean total bilirubin was 5.2 (1.7-32.4); mean alkaline phosphatase was 433.1 (130-1465) and mean γ-glutamyl transpeptidase was 502.7 (92.9-2000.0).

Single duct-to-duct anastomosis was the most common anastomosis type (n = 145, 74.0%). Biliary stricture (n = 91, 46.4%) and a filling defect on an MIP image (n = 90, 45.9%) were the most common findings. Biloma and hematoma were noted in 13 (6.6%) and 10 (5.1%) patients, respectively (Table 2).

The mean anastomosis site angles were 112.9° and 109.2° according to radiologists 1 and 2, respectively, and the ICC was 0.896 (P < 0.001). The lengths of the filling defects on the MIP images were 3.4 mm according to both radiologists, and the ICC was 0.921 (P < 0.001) (Table 3).

Factors predicting biliary intervention

Factors with P values < 0.20 in the univariate analysis were the anastomosis site angle on a 3D image [hazard ratio (HR) = 0.42, 95% confidence interval (CI), 0.27-0.65, P < 0.001], recipient age > 65 years (HR = 2.10, 95%CI: 0.85-5.20, P = 0.110), a filling defect on an MIP image (HR = 2.44, 95%CI: 1.58-3.75, P < 0.001), length of the filling defect on an MIP image

Table 1 Baseline characteristics of the patients *n* (%)

Variable	
Recipient age (yr)[1]	49.7 ± 10.1 (13-68)
Older age patients (> 65 yr)	6 (3.1)
Recipient sex (M/F)	138 (70.0)/58 (30.0)
Donor age (yr)[1]	34.0 ± 10.9 (16-64)
Older donor age (> 60 yr)	2 (1.0)
Donor sex (M/F)	114 (58.2)/82 (41.8)
Age difference (recipient age - donor age)	15.7 ± 14.4 (-22 to 42)
MELD score	17.4 ± 10.4 (2.1 to 58.1)
High score patients (> 35)	13 (6.6)
Cause	
LC-B	51 (26.0)
LC-C	4 (2.0)
Alcohol	27 (13.8)
Hepatocellular carcinoma	80 (40.8)
Combined	5 (2.6)
Hepatitis A	9 (4.6)
Other (drug, autoimmune, unknown)	20 (10.2)
Total ischemic time	91.5 ± 16.0 (60-145)
Group 1[2]	93.7 ± 17.9
Group 2[2]	88.8 ± 15.1
Number of patients with biliary intervention	89 (45.4)
ERCP	38
PTBD	12
Both (ERCP and PTBD)	38
Re-operative intervention	0
Mean duration without biliary intervention (mo)	33.5 ± 28.6 (1-89)

[1]Mean age; [2]The groups were categorized according to the anastomosis site angle (median angle = 113.3°); group 1, angle > 113.3°; group 2, angle ⩽ 113.3. MELD: Model for end-stage liver disease; ERCP: Endoscopic retrograde cholangiopancreatography; PTBD: Percutaneous transhepatic biliary drainage; LC: Liver cirrhosis.

Table 2 Clinical profiles of the patients analyzed *n* (%)

Variable	Number of patients
Using T-tube	13 (6.6)
Anastomosis method	
Type 1[1]	145 (74.0)
Type 2[2]	51 (26.0)
MRI findings	
Filling defect on MIP image	90 (45.9)
Diffuse bile duct dilatation	29 (14.8)
Biliary stricture	91 (46.4)
Biliary leakage	6 (3.1)
Biloma	13 (6.6)
Hematoma	10 (5.1)
Thrombus, infarct	3 (1.5)
Common bile duct stone	2 (1.0)

[1]Type 1, single duct-to-duct anastomosis; [2]Type 2, double duct-to-duct anastomosis including hepatoplasty. MRI: Magnetic resonance imaging; MIP: Maximum intensity projection.

(HR = 1.04, 95%CI: 1.01-1.06, *P* = 0.010), biliary leakage (HR = 2.49, 95%CI: 1.01-6.14, *P* = 0.049), hematoma (HR = 1.80, 95%CI: 0.78-4.10, *P* = 0.179), and diffuse bile duct dilatation (HR = 1.59, 95%CI: 0.93-2.70, *P* = 0.088) (Table 4).

The significant risk factors in the multivariate analysis were a filling defect on an MIP image (HR = 2.18, 95%CI: 1.41-3.38, *P* = 0.001), biliary leakage (HR = 2.52, 95%CI: 1.02-6.20, *P* = 0.048), and the anastomosis site angle (HR = 0.48, 95%CI: 0.30-0.75, *P* < 0.001) (Table 4).

Table 3 Patient characteristics and inter-observer agreement

	Radiologist 1	Radiologist 2	Inter-observer agreement
Anastomosis site angle (°)	112.9 (32.5-168.4)	109.2 (31-173)	0.896 (*P* < 0.001)
Length of filling defect (mm)	3.4 (0-33.9)	3.4 (0-33)	0.921 (*P* < 0.001)

Table 4 Cox regression model for factors predicting biliary intervention

Variable	Univariate		Multivariate	
	Exp (B)	95%CI	Exp (B)	95%CI
Recipient age	1.01	0.99-1.04		
Older age (> 65 yr)	2.10	0.85-5.20		
Recipient sex	1.11	0.69-1.70		
Donor age	1.00	0.98-1.02		
Older donor age (> 60 yr)	0.92	0.13-6.51		
Donor sex	0.84	0.55-1.28		
Age difference[1]	1.01	0.99-1.02		
MELD score[2]	1.00	0.99-1.02		
High MELD score (> 35)[2]	0.97	0.42-2.22		
Anastomosis method[3]				
Type 2 *vs* 1	1.14	0.72-1.80		
T-tube	0.98	0.43-2.24		
MRI findings				
Anastomosis site angle[4]				
Group 2 *vs* group 1[4]	0.42	0.27-0.65	0.48	0.30-0.75
Filling defect[5]	2.44	1.58-3.75	2.18	1.41-3.38
Length of filling defect[5]	1.04	1.01-1.06		
Diffuse bile duct dilatation	1.59	0.93-2.70		
Biliary stricture	1.03	0.68-1.56		
Biliary leakage	2.49	1.01-6.14	2.52	1.02-6.20
Biloma	1.54	0.74-3.19		
Hematoma	1.80	0.78-4.10		
Thrombus, infarct	0.64	0.09-4.59		

[1]Recipient age-donor age; [2]MELD, model for end-stage liver disease; [3]Type 1, single duct-to-duct anastomosis; type 2, double duct-to-duct anastomosis; [4]Checked on the three-dimensional image; the groups were categorized according to the anastomosis site angle (median angle = 113.3°); group 1, angle > 113.3°; group 2, angle ⩽ 113.3°; [5]Checked on the maximum intensity projection image. MRI: Magnetic resonance imaging.

Anastomosis site angle and biliary intervention

Two groups were created according to the median value of the anastomosis site angle (median angle = 113.3°): group 1, angle > 113.3° and group 2, angle ⩽ 113.3°. The biliary intervention rate was significantly lower in group 1 (30.6% *vs* 60.2% in group 2, *P* < 0.001), and the mean time to biliary intervention was longer in group 1 (62.1 ± 4.1 *vs* 38.5 ± 4.2 in group 2, *P* < 0.001) (Table 5). Kaplan-Meier analysis comparing biliary intervention-free survival between groups 1 and 2 revealed higher survival in group 1 (Figure 2).

Table 5 Biliary intervention rate by anastomosis site angle[1]

Variable	Total number	Number of events	Rate of events	*P* value	Mean time interval to events (mo)	*P* value
Group 1	98	30	30.6%	$P < 0.001$	62.1 ± 4.1	$P < 0.001$
Group 2	98	59	60.2%		38.5 ± 4.2	

[1]The groups were categorized according to the anastomosis site angle (median angle = 113.3°); group 1, angle > 113.3°; group 2, angle ⩽ 113.3°.

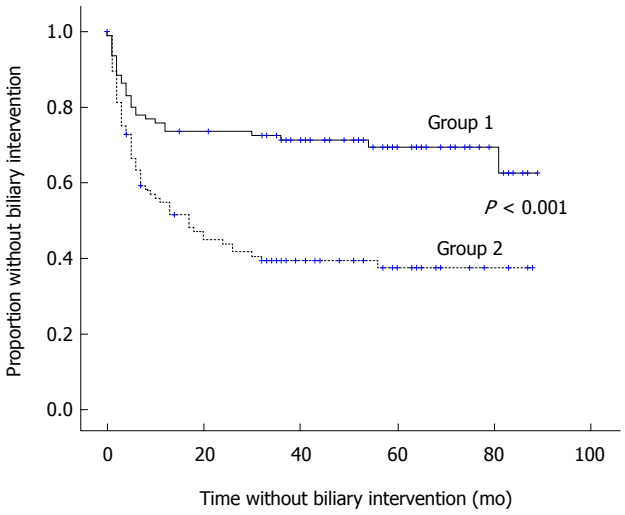

Figure 2 Kaplan-Meier curves for all biliary interventions according to the anastomosis site angle[1]. The Kaplan-Meier curves show that group 1 had a significant survival advantage without biliary intervention compared with group 2 (62.0% *vs* 36.3%, $P < 0.001$). [1]The groups were categorized according to the anastomosis site angle (median angle = 113.3°); group 1, angle > 113.3°; group 2, angle ⩽ 113.3°.

DISCUSSION

Biliary complications after LDLT are an important cause of morbidity and mortality; however, the risk factors for biliary complications that can be determined from an MRCP image after LT have yet to be determined. In the present study, we identified biliary leakage, the presence of a filling defect on an MIP image, and the anastomosis site angle as significant risk factors on MRCP images associated with future biliary intervention.

In our analysis, neither the anastomosis method nor the presence of a T-tube was a risk factor for biliary intervention. Consistent with our results, several studies have demonstrated that the presence of a T-tube is not a risk factor for biliary complications[10,11]; however, some have argued the opposite[2]. Because it is widely accepted to be more physiologically appropriate and has a therapeutic advantage over Roux-en-Y choledochojejunostomy, duct-to-duct anastomosis was performed in our study[12]. We investigated the relationship between increases in the number of duct-to-duct anastomoses and increases in biliary intervention rate. Our findings agree with those of Tsui *et al*[13]; however, they differ from the results of other studies[14,15].

Our results demonstrate that biliary leakage on MRCP was predictive of biliary intervention after LDLT. Biliary leakage can cause complications, such as infection or biliary stricture[16]. Moreover, ERCP and PTBD are the mainstays for managing biliary leakage[17]. Therefore, our result that biliary leakage was a risk factor for biliary intervention is in accordance with previous findings.

A filling defect on MIP images was also a risk factor for biliary intervention. Several studies have indicated the significance of a filling defect detected on MRCP and have suggested that a filling defect could be a sign of bile duct carcinoma or papillomatosis[18-20]. In addition, intra-ductal debris, sludge, and stones could be causes of a filling defect after LT[21]. For these reasons, a filling defect on MIP images should be considered a risk factor for biliary intervention.

However, donor age > 60 years and a MELD score > 35 were not determined to be significant predictors of biliary intervention. Some studies have shown that these are risk factors for biliary complications[5,6]. These inconsistencies could be due to the lower proportion of patients with a donor age > 60 years or a high MELD score (> 35) in our study.

Finally, the anastomosis site angle on a 3D image was shown to be a risk factor for biliary intervention. We demonstrated that a wider anastomosis site angle (*i.e.*, above the median angle of 113.3°) resulted in a lower and delayed incidence of biliary intervention. No study has investigated the relationship between the anastomosis site angle and biliary complications or interventions after LDLT. Thus, we documented, for the first time, that a decrease in the anastomosis site angle on MRCP after LT could be a risk factor for biliary intervention during the follow-up period.

Several limitations of our study should be discussed. First, the design was retrospective. To improve the accuracy of the results, we reviewed every possible factor blinded to biliary outcome. Further prospective studies are warranted to confirm these results. Second, we could not obtain data on patient cold ischemic time, which is a well-known risk factor for biliary complications in deceased donor liver transplantation (DDLT). Generally, however, cold ischemic time is very short during LDLT. Third, the biliary intervention rate we observed in our study was slightly higher than that reported by previous studies. Unfortunately, the reason for the observed high biliary intervention rate remains unknown.

Biliary complications after LDLT are commonly compared with those following DDLT[5]. Thus, pre-

dicting a future need for biliary intervention using a non-invasive method, such as MRCP, could be useful for hematologists and liver transplant surgeons.

In summary, we suggest that MRCP findings of a filling defect on MIP images, biliary leakage, and anastomosis site angle results 1 mo after LDLT can be used to determine the need for future biliary intervention. A further prospective clinical study will be needed to confirm the clinical implications of MRCP 1 mo after LDLT.

COMMENTS

Background

Biliary complications commonly occur after liver transplantation (LT) and are the main cause of morbidity and mortality in LT recipients. Magnetic resonance cholangiopancreatography (MRCP), a non-invasive and accurate tool, is the recommended diagnostic modality for detecting biliary complications after LT. Biliary interventions, such as endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic biliary drainage (PTBD), are generally used to manage these complications. Several investigators have documented risk factors influencing the development of biliary complications. However, no study has investigated the risk factors associated with the future need for intervention based on MRCP findings shortly after living donor LT (LDLT). The purpose of this study was to determine factors, specifically the anastomosis site angle, that increase the requirement for biliary intervention during follow-up in LDLT recipients on MRCP images 1 mo after LT.

Research frontiers

In this study, the authors documented several risk factors for biliary complication by MRCP. They suggest that MRCP findings of a filling defect on maximum intensity projection (MIP) images, biliary leakage, and anastomosis site angle results 1 mo after LDLT can be used to determine the need for future biliary intervention.

Innovations and breakthroughs

No study has investigated the relationship between the anastomosis site angle and biliary complications or interventions after LDLT. Thus, current study documented, for the first time, that a decrease in the anastomosis site angle on MRCP after LT could be a risk factor for biliary intervention during the follow-up period.

Applications

Biliary complications after LDLT are an important cause of morbidity and mortality. Thus, predicting a future biliary intervention using a non-invasive method, such as MRCP, could be useful for hematologists and liver transplant surgeons.

Terminology

MRCP: A non-invasive magnetic resonance imaging tool used to visualize the biliary tract with high sensitivity and specificity. ERCP: An endoscopic procedure specialized for viewing the biliary system and treating biliary complications such as stone and stricture. PTBD: An invasive procedure used to approach the biliary system and treat biliary complications *via* a percutaneous route.

Peer-review

Lee *et al* analyzed MRCP imaging performed 1 mo after LDLT to determine risk factors for biliary intervention. The anastomosis site angle, a filling defect in the anastomosis site, and biliary leakage on MRCP were identified as significant risk factors. Moreover, a narrower anastomosis site angle was related to earlier biliary intervention. For the first time, they showed that the anastomosis site angle may be associated with the need for biliary intervention. A further prospective clinical study will be needed to confirm the clinical implications of MRCP mo after LDLT.

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ORIGINAL ARTICLE

Retrospective Study

Using typical endoscopic features to diagnose esophageal squamous papilloma

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Author contributions: Shih SC and Chen MJ designed the study; Bair MJ performed the pathological analysis; Wong MW and Chen MJ wrote this article; Wang TE and Chang CW prepared the endoscopic photographs and conducted the literature review; Chu CH critically read the manuscript; Wang HY and Shih SC supported this work and supervised the final editing; all authors read and approved the final manuscript.

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Informed consent statement: Patients were not required to give informed consent to the study because the retrospectively analysis used anonymous clinical data.

Conflict-of-interest statement: We have no financial relationships to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM: To better understand some of the superficial tiny lesions that are recognized as squamous papilloma of the esophagus (SPE) and receive a different pathological diagnosis.

METHODS: All consecutive patients with esophageal polypoid lesions detected by routine endoscopy at our Endoscopy Centre between October 2009 and June 2014 were retrospectively analysed. We enrolled patients with SPE or other superficial lesions to investigate four key endoscopic appearances (whitish color, exophytic growth, wart-like shape, and surface vessels) and used narrow band imaging (NBI) to distinguish their differences. These series endoscopic images of each patient were retrospectively reviewed by three

experienced endoscopists with no prior access to the images. All lesion specimens obtained by forceps biopsy were fixed in formalin and processed for pathological examination. The following data were collected from patient medical records: gender, age, indications for esophagogastroduodenoscopy, and endoscopic characteristics including lesion location, number, color, size, surface morphology, surrounding mucosa, and surface vessels under NBI. Clinicopathological features were also compared.

RESULTS: During the study period, 41 esophageal polypoid lesions from 5698 endoscopic examinations were identified retrospectively. These included 24 patients with pathologically confirmed SPE, 11 patients with squamous hyperplasia, three patients with glycogenic acanthosis, two patients with ectopic sebaceous glands, and one patient with a xanthoma. In the χ^2 test, exophytic growth (P = 0.003), a wart-like shape (P < 0.001), and crossing surface vessels under NBI (P = 0.001) were more frequently observed in SPE than in other lesion types. By contrast, there was no significant difference regarding the appearance of a whitish color between SPE and other lesion types (P = 0.872). The most sensitive characteristic was wart-like projections (81.3%) and the most specific was exophytic growth (87.5%). Promising positive predictive values of 84.2%, 80.8%, and 82.6% were noted for exophytic growth, wart-like projections, and surface vessel crossing on NBI, respectively.

CONCLUSION: The use of three key typical endoscopic appearances - exophytic growth, a wart-like shape, and vessel crossing on the lesion surface under NBI - has a promising positive predictive value of 88.2%. This diagnostic triad is useful for the endoscopic diagnosis of SPE.

Key words: Diagnosis; Endoscopy; Esophagus; Squamous papilloma; Narrow band imaging

Core tip: Esophageal superficial and flat lesions possess a wide spectrum of clinical and pathological features. Understanding the endoscopic features of these lesions is essential for their detection, differential diagnosis, and management. Squamous papilloma of the esophagus (SPE) is a rare benign esophageal lesion characterised by whitish and wart-like projections under conventional endoscopy. Overall, 88.2% of patients with polypoid lesions who presented all three typical endoscopic features (exophytic growth, a wart-like shape, and crossing surface vessels) were ultimately histologically confirmed as SPE. Our results may allow endoscopists to more confidently simply observe SPE that meets all endoscopic criteria.

Wong MW, Bair MJ, Shih SC, Chu CH, Wang HY, Wang TE, Chang CW, Chen MJ. Using typical endoscopic features to diagnose esophageal squamous papilloma. *World J Gastroenterol* 2016; 22(7): 2349-2356 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2349.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2349

INTRODUCTION

Esophageal superficial and flat lesions possess a wide spectrum of clinical and pathological features[1]. Understanding the endoscopic features of these lesions is essential for their detection, differential diagnosis, and management. Squamous papilloma of the esophagus (SPE) is a rare benign esophageal lesion characterised by whitish and wart-like projections under conventional endoscopy. The prevalence of SPE ranges from 0.07% to 0.45%[2,3]. Most SPEs are solitary and do not cause symptoms. The sex distribution is either predominantly male or gender neutral[3-6] and the mean age at diagnosis is in the 40s to 50s[3,5-7].

The etiology of SPE is not fully understood and at present, there are at least two etiological hypotheses. One concerns inflammatory reactions such as gastroesophageal reflux disease or a chemical or mechanical mucosal injury[8-10]. The basis of this theory is the high percentage of SPE that occurs at the lower third of the esophagus[2,5,8]. Another hypothesis implicates human papilloma virus (HPV) infection[4,11,12]. However, the exact pathophysiological importance of HPV is not yet clear.

At times, tiny flat lesions that are recognised as SPE due to their small size are not pathologically diagnosed as SPE. Most of these lesions are pathologically diagnosed as squamous cell hyperplasia, glycogenic acanthosis, ectopic sebaceous glands, or xanthomas. The aim of the present study was to clarify the endoscopic characteristics of SPE that contribute to its discrimination from superficial esophageal lesion types.

MATERIALS AND METHODS

Patients

Consecutive outpatients scheduled for esophagogastroduodenoscopy (EGD) for upper gastrointestinal symptoms at our Endoscopy Centre between October 2009 and June 2014 were retrospectively analysed. The following data were collected from patient medical records: gender, age, indications for EGD, and endoscopic characteristics including lesion location, number, colour, size, surface morphology, surrounding mucosa, and surface vessels under narrow-band imaging (NBI). Lesion location was

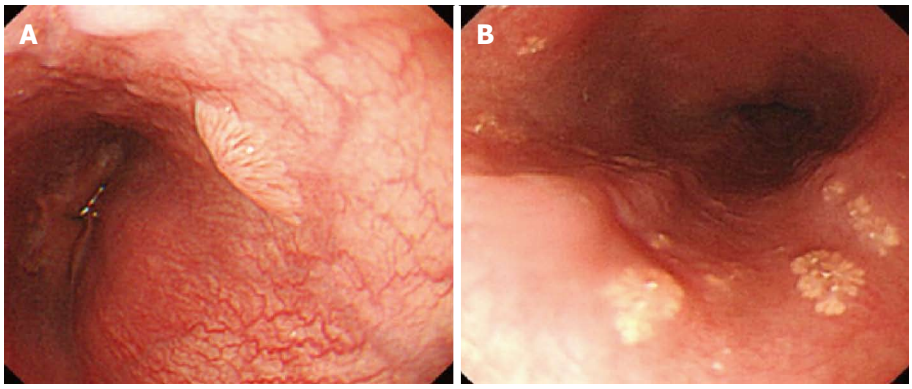

Figure 1 Color of the squamous papilloma of the esophagus looks pale compared to the normal pinkish esophageal mucosa under either near or far focus (A), and the color of the xanthoma looks yellowish under conventional endoscopy (B).

classified as the upper (less than 24 cm from the incisors), middle (between 24 and 32 cm from the incisors), or lower (more than 32 cm from the incisors) oesophagus. Lesion size was estimated using radial jaw biopsy forceps with a width of 6 mm when open (Boston Scientific, MA, United States). The key indications in patients undergoing EGD were epigastric pain, dysphagia, and acid regurgitation or heartburn. This study was approved by the Institutional Review Board of Mackay Memorial Hospital, Taipei, Taiwan (15MMHIS004).

Endoscopic diagnosis

We investigated the diagnostic yield of four key endoscopic characteristics of SPE: whitish colour, exophytic growth, a wart-like shape, and crossing surface vessels under NBI. Series endoscopy (Olympus GIF H260; Olympus Medical Systems Corp, Tokyo, Japan) with an NBI light source was performed in all cases. An evaluation of the entire esophagus was initially performed in a standardised manner by conventional white light endoscopy. Excess mucosal contents were removed using suction and flushes to produce a satisfactory view of the mucosa with no adherent mucus. After turning on the NBI light source, the entire esophagus was carefully scanned by NBI. Lesion specimens obtained by forceps biopsy were fixed in formalin and processed for pathological examination. For patients with multiple SPEs, at least two typical polyps were evaluated histologically. These series endoscopic images of each patient were reviewed by three experienced endoscopists with no prior access to the images. The endoscopists answered yes-or-no questions to determine whether each of the four key endoscopic characteristics was suggestive of an SPE diagnosis. A characteristic was considered positive if two or more endoscopists were in agreement.

Key endoscopic characteristics

Whitish colour was defined as a lesion that looked pale compared to the normal pinkish oesophageal mucosa under either near or far focus (Figure 1A). Exophytic growth was defined as a lesion that not only bulged from the flat mucosa of the esophagus but also floated on the plane without complete attachment to the mucosa (Figure 2A). A wart-like shape was defined as a lesion with an irregular border but with a margin that could be easily distinguished from the normal esophageal mucosa (Figure 3A). To evaluate the fourth characteristic, endoscopy was switched to NBI mode for a more sensitive observation of the blood vessels. Vessel crossing was defined as brownish lines passing through the surface of the lesion (Figure 4A).

Statistical analysis

The size and location of the lesions as well as their endoscopic characteristics (whitish colour, exophytic growth, wart-like shape, and crossing surface vessels under NBI) were statistically analysed to determine the accuracy of SPE diagnosis. Categorical endoscopic data were compared using Fisher's exact test or the χ^2 test, as appropriate. Continuous variables are expressed as the mean ± SD and were compared using the Student's *t*-test. SPSS version 12.0 (SPSS Inc., Chicago, IL, United States) was used for all statistical analyses. All statistical tests were two-tailed, and statistical significance was defined as $P < 0.05$. For SPE and the other diagnostic groups, each characteristic was evaluated independently for sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy.

RESULTS

Basic demographic features

During the study period, 41 patients who were diagnosed with esophageal polypoid lesions from among 5698 endoscopic examinations were retrospectively reviewed. These included 24 (0.42%) patients with pathologically confirmed SPE, 11 patients with squamous hyperplasia, three patients with glycogenic acanthosis, two patients with ectopic sebaceous glands, and one patient with a xanthoma.

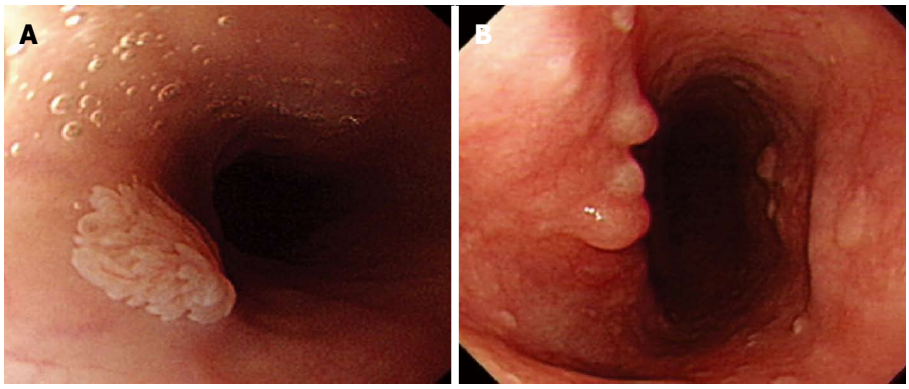

Figure 2 Exophytic growth of squamous papilloma of the esophagus was defined as a lesion that not only bulged from the flat mucosa of the oesophagus but also floated on the plane without complete attachment to the mucosa (A), and SPE may also appear as a sessile polypoid lesion tightly connected to the mucosa (B).

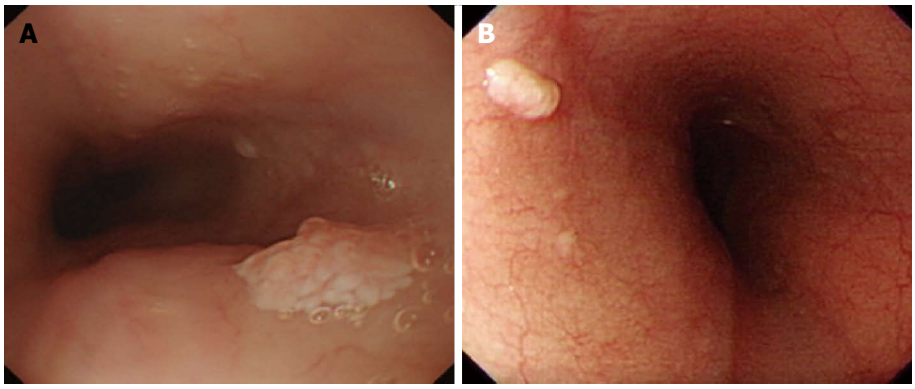

Figure 3 Wart-like shape of squamous papilloma in the esophagus was defined as a lesion with an irregular border but with a margin that could be easily distinguished from the normal esophageal mucosa (A), and the border of squamous hyperplasia was also well-defined and the margin was clear (B).

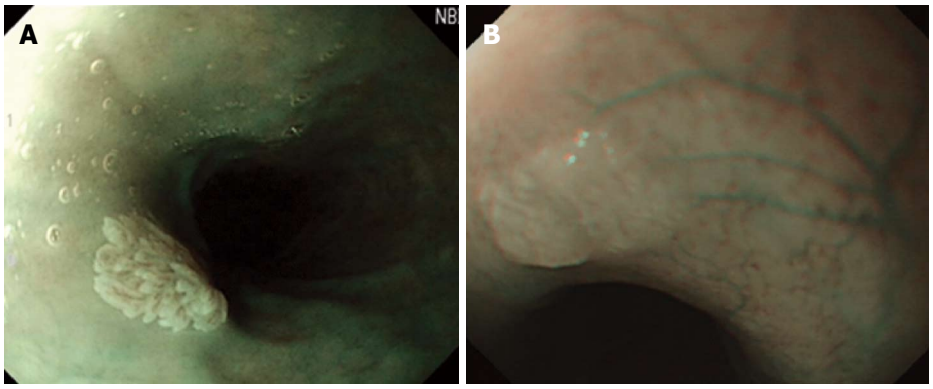

Figure 4 Under UBI mode of endoscopy, vessel crossing was defined as brownish lines passing through the surface of the lesion (A), and by contrast, no brownish lines on glycogenic acanthosis using NBI was observed (B).

The characteristics of all patients are shown in Table 1. The average age of the 24 SPE patients was 48.9 years (range, 28-68 years) and they included more female patients than male patients (20 *vs* 4). The average SPE size was 3.9 mm (range, 2-8 mm). The most common location of SPE was the middle esophagus (57.5%), followed by the lower (26.1%) and upper (17.4%) esophagus. Solitary SPE was present in 22 of the 24 patients. The indications for EGD were epigastric pain in 15 (62.5%) patients, dysphagia in 1 (4.3%) patient, and acid regurgitation or heartburn in 8 (33.3%) patients. With the exception of the gender ratio, there were no significant differences in parameters such as age, lesion location, lesion size, lesion number, or EGD indication between SPE patients and the other diagnostic groups (Table 1).

Table 1 Basic demographic and endoscopic features between squamous papilloma of the esophagus patients and the other diagnostic groups

Characteristic	SPE (n = 24)		Others (n = 17)		P value
Age (yr)	48	(26-68)	53.3	(27-70)	0.197
Gender					0.014
Male	4	16.6%	9	53.9%	
Female	20	82.7%	8	47.1%	
Lesion character					0.383
Location (upp/med/inf)	5/13/6	21/54/25%	10/6/2001	5.9/58.8/35.3%	
Size (mm)	3.8	(2-8)	3.2	(2-5)	0.098
Single lesion	22	92.0%	14	82.4%	0.369
Reason for EGD					0.533
Epigastric pain	15	62.5%	13	76.5%	
Dysphagia	1	4.3%	1	5.9%	
Acid regurgitation and/or heart burn	8	33.3%	3	17.6%	

SPE: Squamous papilloma of the esophagus; EGD: Esophagogastroduodenoscopy.

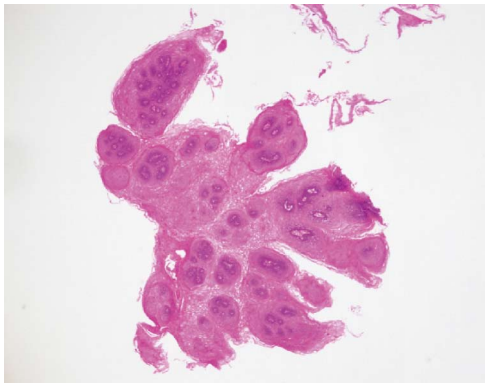

Figure 5 Squamous papilloma of the esophagus is diagnosed pathologically by a hematoxylin and eosin stain. The lesion is composed of papillary fronds lined by several layers of non-keratinizing squamous epithelium and forming finger-like projections (magnification × 20).

Table 2 Each characteristic was evaluated independently for sensitivity, specificity, positive predictive value, negative predictive value, and accuracy in the squamous papilloma of the esophagus and other diagnostic groups (%)

	Sensitivity	Specificity	PPV	NPV	Accuracy
Whitish color	79.2	18.8	59.4	37.5	55.0
Exophytic growth	66.7	81.3	84.2	61.9	72.5
Wart-like projections	87.5	68.8	80.8	78.6	80.0
Surface vessel crossing on NBI	79.2	75.0	82.6	70.6	77.5
Combination of exophytic growth, wart-like shape and surface vessel crossing	62.5	87.5	88.2	60.9	72.5

NBI: Narrow band imaging; PPV: Positive predictive value; NPV: Negative predictive value.

Endoscopic characteristics

Most lesions considered to be SPE were small but recognisable because of margins that were demarcated from the normal esophageal mucosa. We compared the statistical results of each characteristic between SPE and the other lesion types. In the χ^2 test, exophytic growth (P = 0.003), a wart-like shape (P < 0.001), and crossing surface vessels under NBI (P = 0.001) were more frequently observed in SPE than in other lesion types. By contrast, there was no significant difference regarding the appearance of a whitish colour between SPE and other lesion types (P = 0.872).

Pathological characteristics

SPE was diagnosed pathologically by a hematoxylin and eosin stain showing many squamous cells surrounding the vascular connective tissue and forming finger-like projections (Figure 5). By comparison, only an increasing number of squamous cells in the epithelium were noted in squamous hyperplasia. After periodic acid-Schiff staining, glycogenic acanthosis exhibits a combination of cellular hyperplasia and increased cellular glycogen. A xanthoma consists of fat accumulation in foamy histiocytes beneath the squamous epithelium. An ectopic sebaceous gland appears as mucosa covered by benign squamous epithelium with a focal sebaceous cell component. There were no dysplastic or malignant components in these specimens.

Using typical endoscopic appearances to predict SPE

Each characteristic was evaluated independently for sensitivity, specificity, PPV, NPV, and accuracy in SPE and the other diagnostic groups (Table 2). Among the typical endoscopic appearances, the most sensitive characteristic was wart-like projections (81.3%) and the most specific was exophytic growth (87.5%). Promising PPVs of 84.2%, 80.8%, and 82.6% were noted for exophytic growth, wart-like projections, and surface vessel crossing on NBI, respectively. However, fair NPVs of 61.9%, 78.9%, and 70.6% were noted for exophytic growth, wart-like projections, and surface vessel crossing on NBI, respectively. Using the diagnostic triad of exophytic growth, a wart-like shape, and surface vessel crossing under NBI achieved the

highest PPV of 88.2% for SPE.

DISCUSSION

A variety of benign esophageal lesions can be found during endoscopic evaluation. Although they are uncommon, cause no symptoms, and have no malignant potential, such lesions can pose a challenge for establishing an accurate diagnosis and a management plan. This study was the first to distinguish SPE from other tiny flat lesions in the esophagus using typical endoscopic appearances. We concluded that using three key endoscopic features of SPE, namely exophytic growth, a wart-like shape, and vessel crossing, can improve the diagnosis of SPE.

In our study, the average age of the 24 patients with SPE was 48.9 years, which is consistent with the fact that SPE is most commonly diagnosed in patients who are in their 50s[7,8]. Women were extremely dominant in our SPE population, with a male/female ratio of 1/4.75. In previous studies from Italy and other Western countries, most studies had male-dominant populations (male/female: 2.67/1 to 3.4/1)[5,9,11,13] or nearly equal sex distributions (male/female: 1.05/1 to 1/1.21)[2-4,6,14,15]. By contrast, one study in Japan reported a male/female ratio of 1/1.57[6]. Based on our study and the Japanese study, we suggest that there is a geographic difference whereby SPE is more common in women in Asia. Most reports from Western countries indicated a male predominance and a high prevalence of SPE in the lower third esophagus, which may be related to a high prevalence of erosive esophagitis. In our study, more than half of SPE (54.0%) cases occurred in the middle esophagus, similar to the Japanese study that reported a proportion of 52.6%. This suggests that the proposed inflammatory reactions due to gastroesophageal reflux disease may not constitute the main aetiology of SPE in our patients.

HPV infection has been thought to be associated with SPE. The HPV infection rate in the SPE population has been variable (0 to 50%) and the relationship between HPV infection and SPE remains controversial[1,4-6,14]. HPV-positive SPE showed a female predominance, relatively young age, and high prevalence of a middle esophagus location[6]. It is possible that these differences may be related to geographic variability in the frequency of oral HPV infection. We do not know whether or not the female dominance of SPE in our study is related to the prevalence of HPV infection. Further prospective cohort studies would be mandatory to better evaluate the impact of HPV in the pathogenesis of SPE.

On EGD, SPE appear as small, whitish-pink, wart-like exophytic projections that must be differentiated from other similar-appearing lesions such as squamous cell hyperplasia, verrucous squamous cell carcinoma, glycogenic acanthosis, and other rare flat lesions such as heterotopic sebaceous glands or xanthomas. The majority of SPEs are solitary. In our study, all but two patients had multiple SPEs. Glycogenic acanthosis typically appears as multiple lesions that are believed to grow more numerous and larger with age. Xanthoma looks yellowish (Figure 1B) and glycogenic acanthosis often appears paler than the surrounding mucosa. SPE lesions not only bulged from the flat mucosa of the oesophagus, but also floated on the plane without complete attachment to the mucosa. In rare circumstances, however, SPE may appear as a sessile polypoid lesion tightly connected to the mucosa (Figure 2B). The border of SPE was irregular, but the margin could be easily distinguished from the normal esophageal mucosa. The border of squamous hyperplasia was also well-defined and the margin was clear (Figure 3B). SPE is characterised histologically by finger-like tissue projections lined by a core of connective tissue that contains small blood vessels. For this reason, several brownish lines could be observed passing through the surface of the SPE on NBI. By contrast, we observed no brownish lines on glycogenic acanthosis using NBI (Figure 4B).

Although the two lesion types occur in the same esophageal sites and papilloma occurs earlier, the different male-to-female ratios between these two lesion types may indicate that SPE does not share the same risk factors as esophageal squamous cell carcinoma. Most studies indicated that SPE is generally a benign oesophageal lesion. However, there were a few case reports that presented papillomatosis of the esophagus with a squamous carcinoma component[16-20]. One study found synchronic planocellular carcinoma in a patient with SPE[4]. d'Huart *et al*[21] conducted a recent cohort study in France; during a median follow-up period of 21 mo, 2 esophageal squamous cell carcinomas were detected from 78 patients with SPE. The prevalence of associated cancer was 1.3%. One carcinoma was located at the previous resection site of the SPE and the other was located at a different area. In these rare circumstances, SPE or papillomatosis should be surveyed for malignancy.

There were some limitations to the present study. The first is its retrospective design, in that the series endoscopic images of each patient were reviewed retrospectively. We do not know whether the endoscopist could judge these key characteristics accurately in real time. The clinical outcomes should be validated in a prospective study. Second, while we acquired images using multiple angles and focuses, we did not use magnifying endoscopy to obtain more detailed information. We do not know if magnifying endoscopy can provide more relevant key characteristics that would result in better outcomes. Third, all specimens were obtained using cold biopsy forceps. This may be a less accurate method for

pathological evaluation than endoscopic resection.

Overall, 88.2% of patients with polypoid lesions who presented all three typical endoscopic features of SPE were ultimately histologically confirmed as SPE. The advantage of the study is in showing that the benign nature of SPE makes it possible to predict SPE using endoscopic criteria without histological examination. Data from the literature suggest that SPE is a generally benign lesion of the esophagus, except for papillomatosis or a large lesion. Our results may allow endoscopists to more confidently simply observe a single, small lesion in the oesophagus that meets all endoscopic criteria. This prevents unnecessary biopsy, especially for patients undergoing a non-sedative endoscopy that requires them to tolerate a longer biopsy procedure.

COMMENTS

Background

Squamous papilloma of the esophagus (SPE) is a rare benign esophageal lesion characterised by whitish and wart-like projections under endoscopy. However, some superficial tiny lesions that are recognised as squamous papilloma in the esophagus receive a different pathological diagnosis.

Research frontiers

Narrow band imaging is composed of just two specific wavelengths that are strongly absorbed by haemoglobin. The shorter wavelengths (blue band) only penetrate the top layer of the mucosa, while the longer wavelengths (red band) penetrate deep into the mucosa. These wavelengths allow a better understanding of the vasculature of mucosal lesions.

Innovations and breakthroughs

The advantage of the study is in showing that the benign nature of SPE makes it possible to predict SPE using endoscopic criteria without histological examination.

Applications

The use of three key typical endoscopic appearances - exophytic growth, a wart-like shape, and vessel crossing on the lesion's surface under narrow band imaging (NBI) - to predict SPE has a promising positive predictive value of 88.2%. This diagnostic triad is useful for the endoscopic diagnosis of SPE.

Terminology

NBI is a powerful optical image enhancement technology that improves the visibility of blood vessels and other structures on the mucosa.

Peer-review

The authors did a retrospective study to analyse whether some key endoscopic appearances (whitish color, exophytic growth, wart-like shape, and surface vessels) and narrow band imaging can distinguish squamous papilloma from other types of benign lesions in the esophagus. They claimed that combination of three key typical endoscopic appearances-exophytic growth, a wart-like shape, and vessel crossing on the lesion's surface under NBI-has a good positive predictive value, and this triad is useful for the endoscopic diagnosis of squamous papilloma.

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ORIGINAL ARTICLE

Clinical Trials Study

Effects of endoplasmic reticulum stress on the expression of inflammatory cytokines in patients with ulcerative colitis

Nan Li, Xue-Ming Wang, Li-Jun Jiang, Meng Zhang, Na Li, Zhen-Zhen Wei, Nan Zheng, Ya-Jiao Zhao

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Author contributions: Li N designed the research; Wang XM, Jiang LJ and Zhang M performed the research; Li N contributed new reagents or analytic tools; Wei ZZ, Zheng N and Zhao YJ analyzed data; and Li N wrote the paper.

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

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Abstract

AIM: To explore the changes of X-box binding protein 1 splicing (XBP1s) and inflammatory cytokine expression in patients with ulcerative colitis (UC) in response to endoplasmic reticulum stress (ERS).

METHODS: Reverse transcription polymerase chain reaction and quantitative polymerase chain reaction were performed to detect the forms of XBP1s and the expression of interleukin (IL)-2, interferon (IFN)-γ, and IL-17α. Differences between patients with UC and normal subjects were then determined.

RESULTS: Mononuclear cells of the peripheral blood of normal subjects and UC patients with were stimulated with no drugs (control), phytohemagglutinin (PHA), thapsigargin (TG), or both PHA and TG. XBP1s in patients with UC exhibited splicing, which was greater with co-stimulation than single stimulation. Co-stimulation increased the expression level of IL-2, IFN-γ, and IL-17α.

CONCLUSION: The T lymphocytes of both normal subjects and patients with UC responded to ERS by activating the XBP1s-mediated signalling pathway, upregulating the expression of inflammatory cytokines, and increasing the occurrence of inflammation. The mononuclear cells in the peripheral blood of patients with UC were more sensitive to ERS than those in the peripheral blood of normal subjects.

Key words: Ulcerative colitis; Endoplasmic reticulum stress; X-box binding protein 1 splicing

Core tip: Endoplasmic reticulum stress (ERS) can repair stress-induced cell damage and restore normal cell function by the inositol-requiring enzyme 1/X-box binding protein 1 splicing (IRE1/XBP1) signalling pathway. However, the link to ulcerative colitis (UC)

remains unclear. In the present study, we report that T lymphocytes respond to ERS by activating the IRE1/XBP1 signalling pathway, upregulating the expression of inflammatory cytokines, and increasing the occurrence of inflammation. In addition, mononuclear cells in the peripheral blood of patients with UC were more sensitive to ERS than normal subjects.

Li N, Wang XM, Jiang LJ, Zhang M, Li N, Wei ZZ, Zheng N, Zhao YJ. Effects of endoplasmic reticulum stress on the expression of inflammatory cytokines in patients with ulcerative colitis. *World J Gastroenterol* 2016; 22(7): 2357-2365 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2357.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2357

INTRODUCTION

The endoplasmic reticulum regulates protein synthesis and folding and promotes the synthesis of various lipids, such as cholesterol and steroids. Various physiological or pathological states, such as an increase in unfolded proteins, synthesis of misfolded proteins, dysregulation of intracellular calcium ions, and changes in intracellular redox state, can cause the occurrence of endoplasmic reticulum stress (ERS)[1]. ERS can repair stress-induced cell damage and restore normal cell functions through an unfolded protein response. Inositol-requiring enzyme 1(IRE1)/X-box binding protein 1 splicing (XBP1) is an important signalling pathway in ERS; IREI/XBPI can reduce protein synthesis, promote protein degradation, increase chaperone generation, slow down ERS occurrence, and return cellular functions to normal. Under ERS, the IREI/XBPI pathway can activate c-Jun N-terminal kinase (JNK)[2,3] related signalling pathways. As a result, release of cytokines and bio-chemotactic factors is promoted, and the body's natural immune system is activated. Researchers have investigated changes in Toll-like receptors[4] and cytokines as well as the immune system to explain the pathogenesis of inflammatory bowel disease and ulcerative colitis (UC). The IREI/XBPI signalling pathway can conserve the integrity of the intestinal mucosal barrier, maintain the number of intestinal Paneth cells, and promote the secretion of cytokines inside the intestine. In this manner, the intestinal mucosa is protected, and the occurrence of intestinal inflammation is reduced[5-7]. However, studies have yet to describe the effects of ERS on T lymphocytes and the corresponding changes in patients in response to ERS. Hence, we determined whether XBPI influences the incidence of UC by adjusting the acquired immune response.

MATERIALS AND METHODS

Main equipment and reagents

Main instruments: The following instruments were used in this study: low-temperature high-speed centrifuge (Eppendorf, Hamburg, Germany); dry thermostat (Hangzhou ORSUS biotechnology Co, Hangzhou, China); DNA diffusion meter (PE, United States); real-time polymerase chain reaction (PCR) system (Eppendorf); polyacrylamide gel electrophoresis and electrotransferring instrument (Bio-Rad Co., Hercules, CA, United States); inverted fluorescence microscope (Nikon, Tokyo, Japan); CO_2 incubator (Thermo Scientific, Boston, MA, United States); high-speed desktop centrifuge (Eppendorf); low-temperature high-speed desktop centrifuge (Eppendorf); vortex turbulencer (Beijing Tongzheng Biotechnology Co., Ltd., Beijing, China); cryogenic tank (Haier Electric Co., Qingdao, China); chemiluminescence analyser (DLR, USA); temperature controller module (Eppendorf; pure water (Millipore, Billerica, MA, United States); Ultraviolet (UV) spectrometer (Pharmacia, Piscataway, NJ, United States); and agarose gel electrophoresis tank (Beijing Liuyi Instrument plant, Beijing, China).

Reagents: The following reagents were used in this study: chloroform, isopropanol, and ethanol (Beijing Chemical Plant); concanavalin A (ConA) (Sigma, St Louis, MO, Unites States); phytohemagglutinin (PHA) (Sigma); thapsigargin (TG) (Sigma); RPMI-1640 medium (Beijing Neuron biotech Co.); FBS (Gibco, Carlsbad, CA, United States); RNAiso Plus (Takara, Tokyo, Japan); RNA reverse transcription kit (Takara, Japan); real-time kit (Takara, Japan); SYBR (Kangwei Reagent Company, Xinzhuang, China); agarose (GENE Co., Chai Wan, China); Goodview (SBSbBio Genentech, Shanghai, China); and lymphocyte stratification fluid (Sigma).

Experimental methods

Normal control (Ctrl) subjects and patients with UC: The normal Ctrl group comprised 18 healthy volunteers (13 males and five females) aged 25–55 years (with a mean age of 40 years). These volunteers were selected from May 2012 to March 2013 from the Department of Gastroenterology, Hospital of General Staff Headquarters and excluded from the UC diagnostic criteria. All of the normal subjects were screened for high blood pressure, coronary heart disease, cerebrovascular accidents and other cardiovascular diseases, diabetes, thyroid dysfunction, and other endocrine, gastroenterological, and metabolic diseases, liver-kidney-pancreatic diseases, hypoxia-related diseases, inflammation, cancer, and stress response. A total of 21 UC patients aged 23–54 years (with a mean age of 39 years) were selected from May 2012 to March 2013 from the Department of Gastroenterology, Hospital of General Staff Headquarters, Xiyuan Hospital and West division of Chaoyang Hospital. Of the 21 patients, 16 were males and five were females. These patients were diagnosed with moderate to severe UC in accordance with the "consensus of diagnosis and treatment of UC", enacted

Table 1 Clinical data of patients with ulcerative colitis

No.	Gender	Age (yr)	Enteroscopic diagnosis	Pathological appearance
1	M	25	Severe, in acute period	Acute and chronic inflammation
2	M	52	Severe, in acute period	Acute and chronic inflammation
3	F	48	Moderate-severe, in acute period	Chronic and chronic inflammation
4	M	43	Moderate-severe, in remission period	Chronic inflammation
5	F	57	Moderate, in acute period	Chronic inflammation
6	M	50	Moderate, in acute period	Chronic inflammation
7	M	42	Moderate, in remission period	Chronic inflammation
8	M	23	Moderate, in acute period	Acute and chronic inflammation
9	M	41	Moderate -severe, in acute period	Acute and chronic inflammation
10	M	23	Moderate, in acute period	Acute inflammation
11	M	45	Moderate-severe, in acute period	Acute and chronic inflammation
12	M	35	Severe, in remission period	Acute and chronic inflammation
13	M	47	Severe, in acute period	Acute and chronic inflammation
14	M	33	Moderate -severe, in remission period	Chronic inflammation
15	F	56	Moderate, in remission period	Chronic inflammation
16	M	51	Moderate, in acute period	Chronic inflammation
17	M	40	Moderate, in remission period	Acute inflammation
18	M	22	Moderate, in acute period	Acute and chronic inflammation
19	M	39	Moderate-severe, in remission period	Chronic inflammation
20	M	27	Moderate, in acute period	Acute inflammation
21	M	43	Moderate-severe, in remission period	Acute inflammation

in Jinan in 2007. The patient data are shown in Table 1.

Cell isolation and primary culture were performed in accordance with the principles and methods of American Type Culture Collection (ATCC).

Grouping and treatment of human peripheral blood mononuclear cells

Peripheral blood mononuclear cell suspensions were obtained from normal volunteers and patients with UC; these suspensions were then seeded in 12-well plates with 1 mL of the cell suspension in each well. The cell density was adjusted to 5×10^8 cells/L.

The experiment was divided into two groups, namely, the peripheral blood mononuclear cells from normal volunteers and those from patients with UC. Each group was further divided into four subgroups, namely, the Ctrl group, the PHA stimulation group, the TG stimulation group, and the PHA + TG co-stimulation group. Each group was set in triplicate wells, and the experiment was repeated thrice.

Table 2 Primer sequences of human cytokines

Human cytokines	Primer name	Sequence
IL-2	Forward	5′-AAGTTTTACATGCCCAAGAAGG-3′
	Reverse	5′-AAGTGAAAGTTTTTGCTTTGAGCTA-3′
IFN-γ	Forward	5′-AGGGAAGCGAAAAAGGAGTCA-3′
	Reverse	5′-GGACAACCATTACTGGGATGCT-3′
IL-17α	Forward	5′-GAGCCCCAAAAGCAAGAGGAA-3′
	Reverse	5′-TGCGGGCATACGGTTTCATC-3′
IL-4	Forward	5′-GCCAAGACCCCTTCGAGAAAT-3′
	Reverse	5′-CCGTCCCTGTTATCTGCCTCC-3′
GAPDH	Forward	5′-TGTGGGCATCAATGGATTTGG-3′
	Reverse	5′-ACACCATGTTATTCCGGGTCAAT-3′

IL: Interleukin; IFN: Interferon.

Table 3 Primer sequences of X-box binding protein 1 splicing

XBP1	Primer name	Sequence
Human XBP1	Forward	5′-A AAC AG A GTA GCA GCT CAG ACT GC-3′
	Reverse	5′-TC CTT CTG GGT AGA CCT CTG GGA G-3′
Mouse XBP1	Forward	5′-A AAC AG A GTA GCA GCG CAG ACT GC-3′
	Reverse	5′-TC CTT CTG GGT AGA CCT CTG GG

XBP1: X-box binding protein 1 splicing.

Drugs were not added to the Ctrl group. A 20 μL PHA solution was added to the PHA stimulation group at a final concentration of 5 μg/mL. Thirty microliters TG solution was added to the TG stimulation group at a final concentration of 300 nmol/L. Twenty microliters PHA solution and 0 μL TG solution were added to the PHA + TG co-stimulation group. The groups were cultured at 37 ℃ and 5% CO_2 for 12 h; afterwards, the cells were collected to extract RNA for subsequent use.

Real-time polymerase chain reaction

Extraction of total RNA: The total RNA was extracted using the TRIzol method and stored at -70 ℃ for reverse transcription.

Analysis of experimental results: After the reaction, the amplification curve and the melting curve of real-time PCR were confirmed. Meanwhile, the PCR quantitation standard curve was performed. The primer sequences are shown in Table 2.

PCR products were then added onto the agarose gel tank for electrophoresis to observe the splicing of XBP1 among the primer sequences of the groups (Table 3).

Statistical analysis

The experimental data were expressed as mean ±

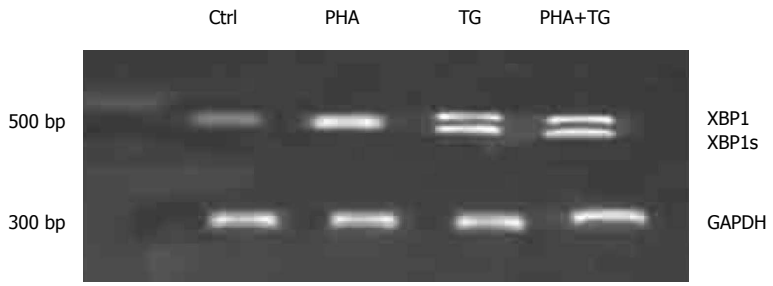

Figure 1 Expression detection of X-box binding protein 1 splicings in the phytohemagglutinin-thapsigargin co-stimulation group. After PHA-TG co-stimulation, the expression of XBPIs in the co-stimulation group was significantly greater than the Ctrl group. PHA: Phytohemagglutinin; TG: Thapsigargin; XBP1: X-box binding protein 1 splicing.

SD, and the statistical software Graphpad Prism 5.0 (La Jolla, CA, United States) was used to plot the data. T tests were performed to determine significant differences between groups; where $P < 0.05$ was considered statistically different and $P < 0.01$ was considered significantly and statistically different.

RESULTS

XBP1s and the expression of inflammatory cytokines in peripheral blood mononuclear cells of normal and UC patients under ERS

Human T lymphocytes were stimulated and activated, and, from the perspective of ERS effects, they produced effects towards the endoplasmic reticulum through XBPI. Under the ERS state, T lymphocytes could stimulate the adaptive immune system through XBPI, thus promoting the expression of inflammatory cytokines.

XBP1s in peripheral blood mononuclear cells of activated UC patients under ERS

The peripheral blood mononuclear cells of UC patients were extracted, stimulated, experimentally grouped, and then divided into the Ctrl group and the PHA stimulation group (with a stimulation concentration of 5 μg/mL). The cells of the TG group were collected 12 h after the stimulation to detect XBPIs. The results are shown in Figure 1. The expression of XBPIs in the PHA-TG co-stimulation group was significantly increased relative to that in the Ctrl group.

The cells were collected from the four groups after 12 h of stimulation, and the expression of interleukin (IL)-2, interferon (IFN)-γ, IL-17α, IL-4, and other cytokines were detected. The results are shown in Figure 2. After TG-PHA co-stimulation, the expression of IL-2, IFN-γ, and IL-17α was significantly increased relative to that in the Ctrl group.

To study the changes of XBP1s and the differences in the expression of inflammatory factors in the peripheral blood mononuclear cells of the healthy Ctrl group and the UC group, additional peripheral blood was extracted from normal volunteers. From this blood, mononuclear cells were extracted, and these cells were stimulated PHA, TG, or both. The experiment was divided into four groups, namely, the Control group, the PHA stimulation group (with a concentration of 5 μg/mL), the TG stimulation group (with a final concentration of 300 nmol/L) and the PHA + TG co-stimulation group. The cells were harvested after 12 h of stimulation to detect the status of XBPIs. The results are shown in Figure 3. XBPIs showed no significant change after PHA-TG co-stimulation.

To study the difference in the expression of inflammatory cytokines in the peripheral blood mononuclear cells of the healthy Ctrl group and the UC group under ERS, we conducted the following experiment. The subjects were divided into two groups: the normal group and the UC patient group. Each group was then further subdivided into four subgroups, namely, the Ctrl group, the PHA stimulation group (with a final concentration of 5 μg/mL), the TG stimulation group (with a final concentration of 300 nmol/L), and the PHA+TG co-stimulation group. The cells were harvested after 12 h of stimulation to detect the expression of 1L-2, IFN-γ, 1L-17α, and other cytokines. The results are shown in Figure 4. The expression levels of IL-2, IFN-γ, and IL-17α in the PHA-TG co-stimulation group were significantly increased compared with the levels found in normal volunteers.

PHA stimulation activated T lymphocytes in the peripheral blood mononuclear cells of normal volunteers and UC patients. When TG stimulation was performed under the ERS status, PHA-TG co-stimulation on the peripheral blood mononuclear cells of UC patients caused significant enhancement of XBP1s compared with the Ctrl group, the PHA stimulation group, and the TG stimulation group. No significant difference in XBP1s was found in the normal population. Meanwhile, PHA-TG co-stimulation of peripheral blood mononuclear cells in normal subjects did not significantly change the expression of IL-2, IFN-γ, and IL-17α. However, the expression of the above cytokines in UC patients was increased with co-stimulation, and the differences were statistically significant, indicating that under the same ERS status, the T lymphocytes in UC patients were much more sensitive towards ERS than normal subjects. UC patients may express more pro-inflammatory cytokines, thus increasing the inflammation response.

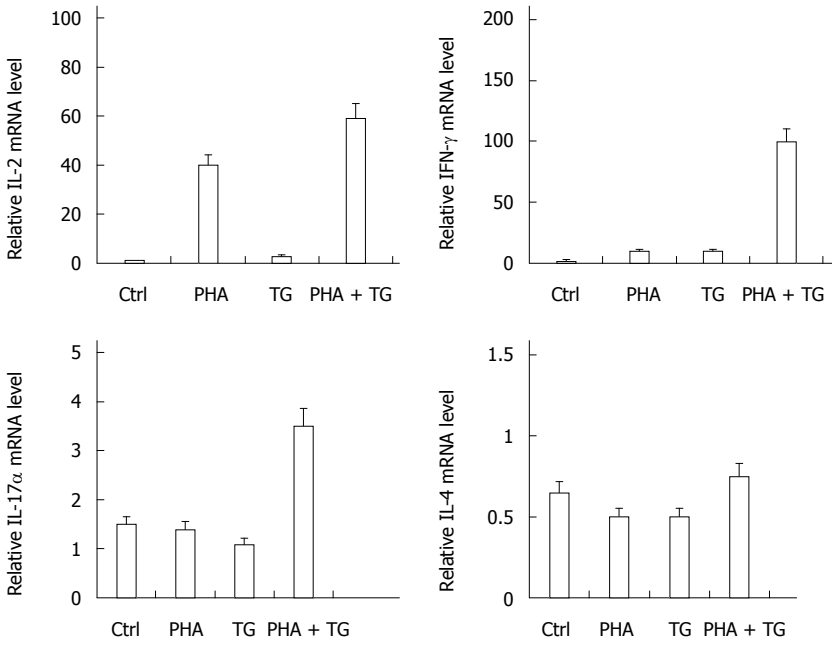

Figure 2 Expression of IL-2, IFN-γ, and IL-17α in the phytohemagglutinin-thapsigargin co-stimulation group. After PHA-TG co-stimulation, the mRNA expression of IL-2, IFN-γ, and IL-17α in the co-stimulation group were significantly increased, while that of IL-4 did not change. PHA: Phytohemagglutinin; TG: Thapsigargin.

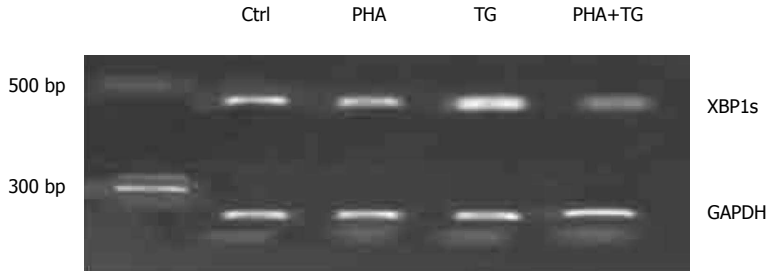

Figure 3 XBPIs detection after phytohemagglutinin-thapsigargin co-stimulation in the peripheral blood mononuclear cells of normal participants. XBPIs showed no significant change after the PHA-TG co-stimulation among the groups. PHA: Phytohemagglutinin; TG: Thapsigargin.

DISCUSSION

Inflammatory intestinal diseases (including UC and Crohn's disease) are chronic non-specific diseases that occur inside the intestine[8]. Currently, the causes are still unclear, although one study has shown that some environmental factors and dietary factors can modulate the clinical course of inflammatory bowel disease (IBD)[9]. Most scholars have classified UC and Crohn' disease as autoimmune diseases[10]. Because the cause of these diseases is unknown, the diagnosis rate is low, and the treatment effects and prognoses are poor. However, the use of biological drugs has opened up new horizons in the management of inflammatory bowel diseases, but the long prognosis remains uncertain[11]. Thus, the problem of diagnosis and treatment continues to be perpetuated. As an organelle of the body, the endoplasmic reticulum plays an important role in the synthesis, maturation, and transport of proteins as well as in the maintenance of calcium homeostasis[12], which affects the folding, quality control, and transport regulation of proteins[13]. Any changes of *in vitro* and *in vivo* conditions affect the functions of the endoplasmic reticulum, thus blocking protein processing and causing a large number of folded proteins to accumulate inside the endoplasmic reticulum, leading to endoplasmic reticulum dysfunction[14]. To ease the pressure of the endoplasmic reticulum, the chaperone binding protein (BiP) dissociates from three important signalling molecules PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring 1 (IRE1) inside the ERS pathway[13,15-17]. Normally, BiP directly acts on the folding protein to promote protein folding and input the synthesised proteins into the endoplasmic reticulum. BiP also releases the pressure of the endoplasmic reticulum, thereby returning endoplasmic reticulum functions back to normal. If the folded proteins of the endoplasmic reticulum were continuously generated at an enormous rate, the pressure would be sustained and the loading would be increased. The PERK,

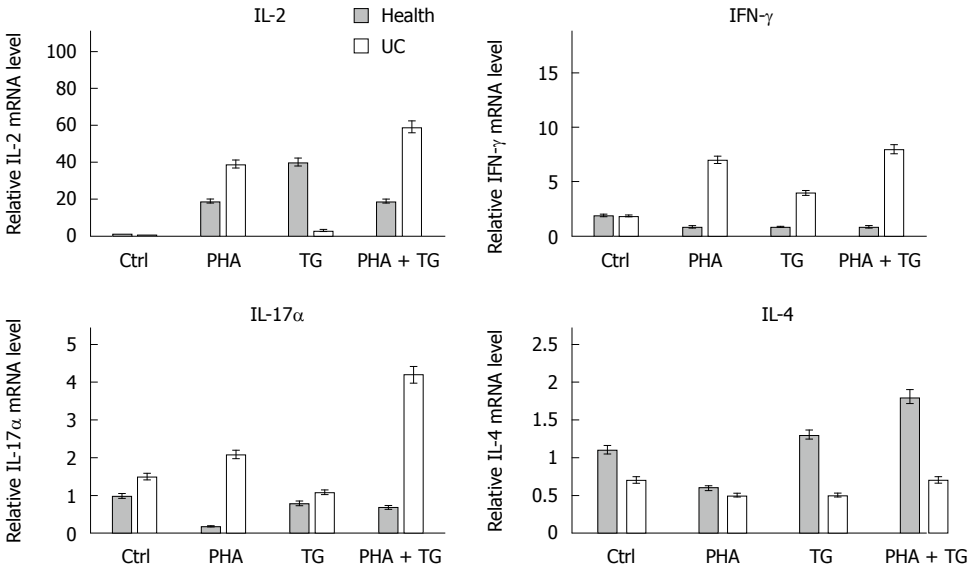

Figure 4 Comparison of the expression of IL-2, IFN-γ, and IL-17α in the peripheral blood mononuclear cells of normal healthy people and ulcerative colitis patients after phytohemagglutinin and thapsigargin stimulation. The mRNA expression levels in the normal healthy people and ulcerative colitis (UC) patients all exhibited changes after TG and PHA + TG stimulation, while the expression of IL-2, IFN-γ, and IL-17α in the peripheral blood mononuclear cells of UC patients was higher than that in normal volunteers. PHA: Phytohemagglutinin; TG: Thapsigargin.

ATF6, and IRE1 pathways would be activated and would reduce the synthesis of proteins, promote the degradation of proteins, and increase the generation of BiP, thus slowing the occurrence of ERS. However, if the pressure load inside the endoplasmic reticulum is not lifted, it would eventually lead to apoptosis[18]. During the occurrence of ERS, the immune system, the channels associated with inflammation and pressure, and the oxidative stress pathways are affected; hence, a number of chronic metabolic diseases and autoimmune diseases, such as type 2 diabetes, fatty liver, neuropathic lesions and various tumours and inflammatory intestinal diseases, would be induced[19]. Recent studies have linked ER stress and the UPR to IBD[20].

Experiments have shown that under ERS, the IRE1/XBP1 pathway could activate the body's natural immune system through TLR (Toll-like receptors)[4]. On the one hand, the activation of XBP1 within macrophages could promote the induction of NOX2 NADPH oxidase by TLR[21]. On the other hand, it could activate the ROS system[22], thus causing the activation of XBP1. This activation would ultimately activate NF-KB, JNK, and other signalling pathways[2,23,24], promote the secretion of IL-6, TNFα, and other cytokines, and act in the early stage of immune response[22]. Under ERS, the expression of XBP1s significantly increased after activation of human T lymphocytes[25]; the expression of IL-2, IFT-γ, and IL-17α increased, and inflammation increased. Compared with inactivated T lymphocytes, the activated T lymphocytes exhibited a significant enhancement of XBP1s after TG stimulation. XBP1s is the active form of XBP1. When the IRE1/XBP1 signalling pathway was activated, XBP1s was significantly enhanced[26]. In comparison with T lymphocytes activated by single stimuli, the mRNA expression levels of IL-2, IFN-γ, and IL-17α was significantly increased after the co-stimulation of the activator and TG[27-29]. Collectively activated T lymphocytes affect ERS by XBP1, namely; under ERS, XBP1 could not only activate the innate immune response[30] but also activate the body's acquired immune response and release cytokines. Acquired immune response is one of the most important parts of the immune system, playing a key role in the body's processing of the immune response. Acquired immunity participates in and affects the occurrence and development of various autoimmune diseases, and influences their prognosis. Previous studies found that the incidence of inflammatory intestinal diseases was most closely related to the autoimmune system. The TLR family was shown to regulate T cells through 15 natural immunity and $CD4^+CD25^+$[31] and to play important regulatory roles in the pathogenesis of IBD by adaptive immunity. Some cytokines, such as TNFα, IL-17α, IL-23, and TGF-β, play important roles in the pathogenesis of inflammatory intestinal diseases, though the inflammatory intestinal diseases are still not clearly understood. In this study, key molecules in the three channels of endoplasmic reticulum were detected. The expressions of IRE1/XBP1 and ATF6 in the intestinal epithelial mucosa of patients with severe UC were much higher than expressions in the normal volunteers, while the expression of PERK was reduced[32]. ERS occurred in patients with inflammatory intestinal disease, while the specific mechanism was still unclear.

Our experiment was designed and conducted based on ERS activation of the acquired immune system in UC patients, and it role in the occurrence

and development of UC. We sampled the peripheral blood of UC patients, separated the mononuclear cells, and then stimulated them with PHA to activate the T lymphocytes. Meanwhile, the TG stimulation was also performed to detect the conditions of XBP1s. Under ERS, UC patients exhibited obvious XBP1s inside the activated T lymphocytes, which was significantly enhanced compared with the unstimulated group and the PHA or TG single-stimulation group. We also detected the cytokines of each group after treatment and found that the mRNA expression of IL-2, IFN-γ, and IL-17α in the peripheral blood mononuclear cells of UC patients was significantly increased after co-stimulation by PHA and TG. We also isolated the peripheral blood mononuclear cells from healthy subjects and stimulated them with PHA, TG, or a combination of both. The results showed that after co-stimulation, the expression of IL-2, IFN-γ, and IL-17α, as well as XBP1s was increased. However, compared with the UC patients, the expression of cytokines and XBP1s in the other groups was significantly reduced. Thus, we believe that the peripheral blood mononuclear cells of UC patients were much more sensitive to ERS. Under ERS, the expression of XBP1s in the peripheral blood mononuclear cells of activated UC patients was much more obvious and could activate the body to achieve the acquired immune response to promote the expression of cytokines, such as IL-2, IFN-γ and IL-17α, aggravating the occurrence of UC.

The activation of T lymphocytes and peripheral blood mononuclear cells in UC patients by TG stimulation significantly increased the expression of IL-2, IFN-γ, and IL-17α. The expression of IL-14, however, was not significantly changed. T helper (Th) cells inside the body's T cells were divided into two subtypes (Th1 and Th2), and IL-2 and IFN-γ were expressed by Th1-type cells, while IL-4 was expressed by Th2 cells. Under normal circumstances, Th1yu and TH2 exist in dynamic equilibrium. The expression of IL-2, IFN-γ, and IL-17α increased, while IL-4 was not increased, indicating that under ERS, lymphocytes drifted towards Th1. The Th1 cells mainly mediate the cellular immune response, and are thus involved in inducing the occurrence of organ-specific autoimmune diseases. This observation further indicated that the body was more likely to develop autoimmune diseases under ERS, which might be an important mechanism for the occurrence of UC. Under ERS, the expression of IL-2, IFN-γ, and IL-17α in the lymphocytes of UC patients were increased, while the expression of IL-4 showed no significant change, indicating that the lymphocytes in UC patients drifted towards TH1; no such phenomenon occurred in healthy people, which is consistent with literature[33].

IL-17α is a cytokine secreted and released by the TH17 subgroup. As a pro-inflammation cytokine, IL-17α often plays roles in autoimmune and infectious diseases[34]. The expression of IL-17α was significantly increased during the process of UC[35]. We used CT to stimulate the peripheral blood mononuclear cells of normal subjects and activated UC patients and found that compared with normal people, the expression of IL-17α was increased in the lymphocytes of UC patients. In addition, the expression of IL-17α increased by the PHA–CG co-stimulation was significantly higher than PHA stimulation alone. We used TG to stimulate the activated human T lymphocytes, and the expression of IL-17α was significantly increased, indicating that ERS could activate T lymphocytes to express IL-17α, thus aggravating inflammation. We used TG to stimulate the activated lymphocytes in UC patients, and the expression level was significantly higher than that in PHA or TG stimulation alone, indicating that UC patients were much more sensitive to ERS.

In summary, under ERS, activated human T lymphocytes could affect the endoplasmic reticulum and promote the expression of inflammatory cytokines through XBP1s, and the lymphocytes drifted towards Th1. Therefore, we considered that under ERS, the acquired immune response could be activated, thus promoting the expressions of inflammatory cytokines and aggravating the occurrence of autoimmune diseases. UC is considered an autoimmune disease. Compared with normal healthy individuals, the peripheral blood lymphocytes of UC patients were much more sensitive to ERS. The lymphocytes could respond to ERS through XBP1s, thus activating the body to achieve the acquired immune response, promoting the expressions of IL-2, IL-17α, and IFN-γ. This response might contribute to the pathogenesis of UC; blocking this pathway might be one way to treat UC, although it still needs to be confirmed by further experiments.

In conclusion, this study found that human T lymphocytes responded to ERS through XBP1s and promoted the secretions of IFN-γ, IL-17α, and IL-2. In addition, we also found that the peripheral blood mononuclear cells of UC patients were much more sensitive to ERS. The T lymphocytes of the patient could respond to ERS through the IRE1/XBP1 pathway, activate the body to obtain the acquired immune response, and promote the expression of inflammatory cytokines. Hence, T lymphocytes play important roles towards the occurrence and development of inflammation.

COMMENTS

Background

Various physiological or pathological states can cause the occurrence of endoplasmic reticulum stress (ERS); ERS can repair stress-induced cell damage and restore normal cell functions through an unfolded protein response. Inositol-requiring enzyme 1(IRE1)/X-box binding protein 1 splicing (XBP1) is an important signalling pathway in ERS. Although the IREI/XBPI signalling pathway can conserve the integrity of the intestinal mucosal barrier, maintain the number of intestinal Paneth cells, and promote the secretion of cytokines inside the intestine, no studies have yet described the effects of ERS on T lymphocytes and the corresponding changes in patients in response to

ERS. Hence, the authors determined whether XBPI influences the incidence of UC by adjusting the acquired immune response.

Research frontiers

Experiments showed that under ERS, the expression of XBP1s is significantly increased after human T lymphocytes were activated and that the IRE1/XBP1 pathway could activate the body's natural immune system through TLR. This activation would ultimately activate NF-κB, JNK, and other signalling pathways, promote the secretion of IL-6, TNFα, and other cytokines, and act in the early stage of immune response.

Innovations and breakthroughs

This study provided direct evidence that T lymphocytes of patients with UC respond similarly to those of normal subjects. The response was through the XBP1s-mediated signalling pathway and was associated with activation of the expression of inflammatory cytokines and increased occurrence of inflammation. In contrast, the mononuclear cells of peripheral blood of UC patients were more sensitive to ERS than the normal subjects.

Applications

Mononuclear cells of peripheral blood may be a possible target in the treatment of UC.

Terminology

ERS, the stress status of the endoplasmic reticulum, repairs stress-induced cell damage and restores normal cell function. IRE1/XBP1 are two kinds of proteins that promote the pathway of ERS repair.

Peer-review

In this interesting study, Nan *et al* explored changes in XBP1s forms and the expression of inflammatory cytokines in patients with UC towards ERS. The results of this study are good.

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ORIGINAL ARTICLE

Observational Study

Endoscopic dilation of complete oesophageal obstructions with a combined antegrade-retrograde rendezvous technique

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Author contributions: Sulz MC designed the study together with Bertolini R; all authors provided data; Sulz MC, Bertolini R, Putora PM, Albrecht F and Broglie MA analysed and interpreted the data and wrote the manuscript; Meyenberger C and Stoeckli SJ interpreted the data and revised the manuscript; all authors approved the final version of the manuscript.

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Abstract

AIM: To investigate the combined antegrade-retrograde endoscopic rendezvous technique for complete oesophageal obstruction and the swallowing outcome.

METHODS: This single-centre case series includes consecutive patients who were unable to swallow due to complete oesophageal obstruction and underwent combined antegrade-retrograde endoscopic dilation (CARD) within the last 10 years. The patients' demographic characteristics, clinical parameters, endoscopic therapy, adverse events, and outcomes were obtained retrospectively. Technical success was defined as effective restoration of oesophageal patency. Swallowing success was defined as either percutaneous endoscopic gastrostomy (PEG)-tube independency and/or relevant improvement of oral food intake, as assessed by the functional oral intake scale (FOIS) (≥ level 3).

RESULTS: The cohort consisted of six patients [five males; mean age 71 years (range, 54-74)]. All but one patient had undergone radiotherapy for head and neck or oesophageal cancer. Technical success was

achieved in five out of six patients. After discharge, repeated dilations were performed in all five patients. During follow-up (median 27 mo, range, 2-115), three patients remained PEG-tube dependent. Three of four patients achieved relevant improvement of swallowing (two patients: FOIS 6, one patient: FOIS 7). One patient developed mediastinal emphysema following CARD, without a need for surgery.

CONCLUSION: The CARD technique is safe and a viable alternative to high-risk blind antegrade dilation in patients with complete proximal oesophageal obstruction. Although only half of the patients remained PEG-tube independent, the majority improved their ability to swallow.

Key words: Oesophageal obstruction; Rendezvous technique; Combined antegrade-retrograde endoscopic dilation; Endoscopic dilation; Head and neck cancer; Radiotherapy

Core tip: Complete obstruction in the proximal oesophagus is rare after radiotherapy for head and neck cancers. We present our institutional experience with endoscopic rendezvous dilation and the clinical outcomes. This technique offers a safe and viable alternative to high-risk blind antegrade dilation. In our series, the rate of technical success was high. Although half of the patients remained percutaneous endoscopic gastrostomy-tube dependent, the majority showed relevant improvement in their ability to swallow and, consequently, in their quality of life.

Bertolini R, Meyenberger C, Putora PM, Albrecht F, Broglie MA, Stoeckli SJ, Sulz MC. Endoscopic dilation of complete oesophageal obstructions with a combined antegrade-retrograde rendezvous technique. *World J Gastroenterol* 2016; 22(7): 2366-2372 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2366.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2366

INTRODUCTION

Complete obstruction in the proximal oesophagus is a rare but severe complication after radiotherapy in head and neck cancer patients[1-3]. Less common causes are gastro-oesophageal reflux disease, Plummer-Vinson-Syndrome, or caustic injury[4-7]. Antegrade reopening and dilation of a complete oesophageal obstruction is difficult and carries a high risk of oesophageal perforation. A combined antegrade-retrograde endoscopic rendezvous procedure offers better visualisation and safer dilation. The antegrade-retrograde rendezvous technique was first described by van Twisk *et al*[8] in 1998, followed by Bueno in 2001[9]. This technique is also termed combined antegrade-retrograde endoscopic dilation (CARD). Several other small series[10-14] were subsequently reported. Most series reported on the technical feasibility of the procedure, but rarely on the functional assessment of swallowing. Therefore, our primary aim was to describe the swallowing outcome, including the dependency on a percutaneous endoscopic gastrostomy (PEG)-tube, using the objective functional oral intake scale (FOIS) to assess patients undergoing this rare endoscopic treatment.

MATERIALS AND METHODS

Patients and endoscopic technique

This single-centre case series included a consecutive cohort of patients who underwent CARD for complete obstruction in the area of the pharyngoesophageal segment at the Cantonal Hospital, St. Gallen, Switzerland, between July 2005 and February 2015. Prior to the intervention, all patients were completely unable to swallow and PEG-tube dependent. The diagnosis of complete obstruction was confirmed by endoscopic and/or radiologic findings. Data extracted included demographic characteristics, clinical parameters, endoscopic therapy, adverse events, and outcome. Approval for using pseudoanonymized patient data was obtained from the local ethics committee and all study participants, or their legal guardian, provided informed written consent prior to study enrollment.

The procedures were performed jointly by experienced endoscopists and head and neck surgeons with the patient under general anaesthesia. The existing PEG-tube gastrostomy is a condition to get access to the stomach for the *retro*grade part of this rendezvous procedure. The first step was to remove the PEG-tube carefully and to keep access to the stomach with a guidewire through the gastrostomy. Then, dilation of the gastrostomy with Savary bougies (Cook Medical, United Kingdom) up to 12 mm over the guidewire was necessary to pass the endoscope. In our experience, balloon dilation was not effective. All *retro*grade endoscopies were performed with extra-slim nasal endoscopes (Olympus, Tokyo, Japan) (Figure 1). Antegrade endoscopy was performed transorally with rigid and/or flexible endoscopes (left to the discretion of the head and neck surgeon). The obstruction within the oesophagus was identified with both endoscopes (Figure 2). Fluoroscopy on the one hand and antegrade transillumination on the other hand are important tools to guide the retrograde and the antegrade endoscope towards each other. Fluoroscopy helps to estimate the length of the obstructed oesophagus and the direction of both endoscopes. Furthermore, the retrograde puncture of the complete obstruction was directed by transillumination (Figure 3). It was crucial to use the hard end of the wire, as it is impossible to succeed puncture of the completely obstructed oesophagus with the soft tip of the wire. The puncture procedure

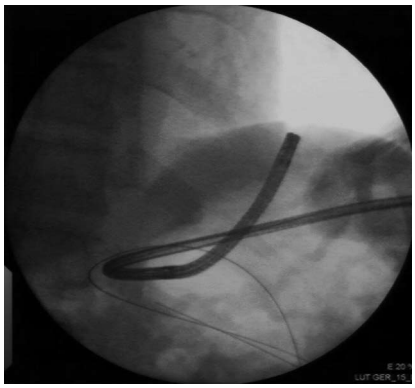

Figure 1 Fluoroscopy. Gastroscope in the stomach, inserted through the existing gastrostomy and used for retrograde puncture.

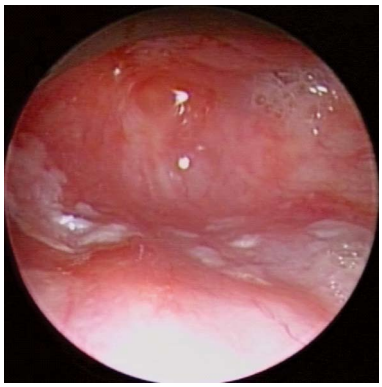

Figure 2 View from the antegrade site. Complete obstruction in the proximal oesophagus.

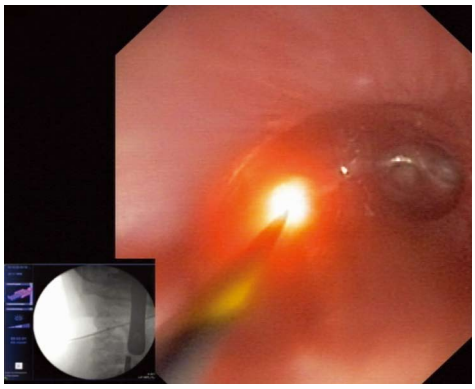

Figure 3 Rendezvous. Retrograde view, transillumination from the proximal site before puncture of the complete obstruction with a VisiGlide guidewire (Olympus, Tokyo, Japan). Inlet: Fluoroscopy showing the antegrade laryngoscope and the retrograde gastroscope opposite each other.

was documented by endoscopy and fluoroscopy (Figure 3). After successful puncture the wire was passed through the obstruction and finally picked up by the operator performing the antegrade endoscopy. Then, dilation was started using small Savary bougies (Cook Medical, United Kingdom) from the antegrade site, followed by insertion of a nasogastric tube to maintain the patency and balloon dilations (Figure 4). Nasogastric tubes were re-inserted after subsequent

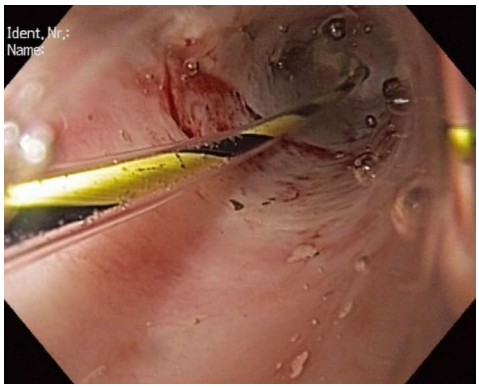

Figure 4 Balloon dilation of the punctured obstruction. A Hercules balloon, 6 mm, was used.

Table 1 Functional oral intake scale items[15]

Level 1	Nothing by mouth
Level 2	Tube dependent with minimal attempts of food or liquid
Level 3	Tube dependent with consistent oral intake of food or liquid
Level 4	Total oral diet of a single consistency
Level 5	Total oral diet with multiple consistencies, but requiring special preparation or compensations
Level 6	Total oral diet with multiple consistencies without special preparation, but with specific food limitations
Level 7	Total oral diet with no restrictions

dilations until the dilated oesophageal lumen was large enough, according to the discretion of the operators. After the procedure a new PEG-tube was placed percutaneously to provide additional nutritional support. Video 1 summarizes the single steps of the procedure. The details of the procedure were not based on a strict protocol. The endoscopists were allowed to choose any available endoscopic device to succeed.

Outcome parameters

Technical success was defined as effective restoration of oesophageal patency. The swallowing success was defined as either PEG-tube independency and/or relevant improvement of oral food intake, as assessed by the FOIS, a 7-point ordinal scale documenting the functional level of oral intake of food and liquids[15]. The scale focuses on the patient's intake by mouth on a daily basis (Table 1). Though the *pre*-therapeutic FOIS level was not assessed formally, the score of all patients corresponded to a FOIS level 1 (nothing by mouth, PEG-tube dependent) as they were unable to swallow anything due to complete obstruction. Relevant improvement of swallowing (FOIS $\geqslant$ level 3) at the time of follow-up was defined as swallowing success.

RESULTS

Patients' characteristics

The cohort consisted of six patients [five males; mean

Table 2 Patients' characteristics and outcomes

No	Sex	Age	Diagnosis	Prior radiotherapy (max dose i. Gy)	Surgery	Chemotherapy	Technical success	FOIS	Notes
1	F	68	Hypopharyngeal carcinoma T3 N0 and oropharyngeal carcinoma T2 N0	68	No	Yes	Yes	6	
2	M	59	Complete occlusion of sinus piriformis after Lyell syndrome	No	No	No	Yes	6	
3	M	54	Squamous carcinoma of the cervical oesophagus cT3 cN2 cM0	66	No	Yes	No	-	Died before follow-up with PEG-tube
4	M	74	Proximal oesophageal carcinoma and carcinoma of the glottic larynx cT2 cN0	Glottis: 66 Oesophagus: 68	Yes	Yes	Yes	-	Died before follow-up with PEG-tube
5	M	68	Carcinoma of the hypopharynx initial cT2c No cM0	70	Yes	No	Yes	2	
6	M	71	Carcinoma of the larynx cT3 cN2	68	No	No	Yes	7	

FOIS: Functional oral intake scale.

Table 3 Technical details and clinical success of endoscopic rendezvous dilation in patients with complete obstruction of the proximal oesophagus

Type of antegrade endoscope	
Rigid, flexible	4, 2
Method of retrograde puncture of the obstruction	
VisiGlide guidewire	4/6
Argon beamer	1/6
First stricture dilation	
With Savary bougie	5/5
Size	median 10 mm (range 6 - 10 mm)
Subsequent dilations before discharge	
With balloon	3/5 patients
size range	15-16.5 mm
With bougie	2/5 patients
size range	12-15 mm
Success rate of rendezvous procedures	
Technical success	5/6 (83%)
Need for recurrent dilations after discharge	5/5
Swallowing success	
Time of follow-up, median (range)	27 mo (2-115)
Need for long-term PEG-tube	3/6
Functional oral intake	
Tube dependent with minimal attempts of food or liquid (level 2)	1
Total oral diet with multiple consistencies without special preparation, but with specific food limitations (level 6)	2
Total oral diet with no restrictions (level 7)	1
Complications of rendezvous procedures	
Mediastinal emphysema (no surgery needed)	1/6
Death	0/6

FOIS: Functional oral intake scale.

age 71 years (range, 54-74)]. Patients' characteristics are shown in Table 2. Two patients died due to their underlying tumour disease 4 and 20 mo after the endoscopic rendezvous intervention. Death was associated with cancer.

Procedure characteristics

Technical details concerning the endoscopic rendezvous intervention are summarized in Table 3. Rigid endoscopes were used in four of six patients by head and neck surgeons; while flexible instruments were applied in two patients for the antegrade access. The retrograde puncture of the obstruction was achieved with a VisiGlide guidewire (Olympus, Tokyo, Japan) in four of six cases (Figure 3). In each case, once a lumen was established, a wire was cautiously passed through, and dilation (median 10 mm, range, 6 - 10 mm) was performed with Savary bougies (Cook Medical, United Kingdom). After the first intervention, nasogastric tubes were inserted in all cases to keep the dilated obstruction open and a new PEG-tube was placed successfully in all patients.

Outcome

Technical success: Technical success was achieved in five out of six patients (Tables 2 and 3). In one patient with squamous carcinoma of the proximal oesophagus, the complete obstruction in the proximal oesophagus could not be punctured retrogradely by VisiGlide wire, super stiff wire, Savary wire, or argon beamer. All five patients with successful puncture were treated with Savary bougies [median size of first dilation 10 mm (range, 6-10 mm)]. However, all five patients with technical success needed subsequent dilations; three were treated with balloons (size range, 15-16.5 mm; Figure 4) and the other two patients with bougies (size range, 12-15 mm) (Table 3). After discharge, repeated dilations or stenting were performed in all five patients during long-term follow-up; however the number of interventions and the time interval varied significantly between the individual patients, depending on their symptoms.

Swallowing success: At the date of follow-up (median 27 mo, range, 2-115 mo), the FOIS results of all patients who survived were available (Table 3). Before the procedure, all patients were completely unable to swallow correlating with FOIS level 1: Nothing by mouth, dependent from PEG-tube feeding. After the endoscopic treatment, containing the initial rendezvous procedure and the following dilations,

in three patients clinical success with a FOIS score more than 6 was reached. Unfortunately, two patients died before the authors could draw any conclusion regarding swallowing during the follow up. One patient had a poor result with a FOIS level of 2 and did not reach swallowing success (defined as FOIS ⩾ 3).

Adverse events: One patient developed a mediastinal emphysema following CARD, without a need for surgery. There were no deaths associated with the endoscopic procedure (Table 3).

DISCUSSION

We present a retrospective single-centre case series of all patients over the last 10 years who were treated with endoscopic antegrade-retrograde rendezvous dilation for complete obstruction in the proximal oesophagus or hypopharynx. The majority of patients had undergone previous radiotherapy, for treatment of head and neck and proximal oesophagus cancers, which is a major cause of stricture or obstruction in the proximal oesophagus[1-3]. Pharyngoesophageal stricture or stenosis necessitating dilations have been described in up to one fifth of patients after radio(chemo)therapy for head and neck cancer[16,17]. A correlation has been described between radiation stricture induction and radiation dose, as well as volume of irradiation to organs at risk (*e.g.*, upper oesophagus)[18]. Complete obstruction may also occur in a minority of affected patients[2].

While intensity modulated radiotherapy better spares the parotid gland and reduces xerostomia rates[19], a review of published literature suggested there is a higher rate of oesophageal stricture with this treatment compared with 3D conventional radiotherapy[20]. Chen *et al*[21] demonstrated that when planning target volumes were reduced (from 5 to 3 mm) and there was daily imaging of the application of radiotherapy treatment, the rate of radiation-induced oesophageal stricture was reduced from 14% to 7%. There is currently a stronger focus on sparing the dose to the pharyngeal constrictor muscles and the cervical oesophagus when feasible; thus, lower rates of (pharyngo-) oesophageal stricture might be expected in the future as a side-effect of radiotherapy.

This is one of the first series presenting clinical outcome data based on assessment of the patients' oral ingestion ability after rendezvous treatment using a validated score. Grooteman *et al*[22] used the Dakkak and Bennett score. Goguen *et al*[23] reported the outcome in terms of the achieved diet and the PEG-tube dependency, and the clinical outcome was recorded by the swallow therapists. The limited data on this topic illustrate that professional medical teams who take care of patients suffering from dysphagia after head and neck surgery or radiotherapy in the head and neck region (*e.g.*, speech therapists, head and neck surgeons, medical radio-oncologists, and gastroenterologists) rarely assess and publish the patients' functional oral food intake using objective scores of their daily routine. In our study, the patients' abilities to intake food orally were assessed using the FOIS. This tool was initially designed to document changes in functional oral intake of food and liquid in stroke patients[15]. The scale is useful to document a clinical change, for example before and after an intervention, such as with speech therapy[15,24].

Other scales are available, such as the Mann Assessment of Swallowing Ability, Acute Stroke Dysphagia Screen, and the Dysphagia Outcome and Severity Scale. However, they are often disease-specific. The FOIS offers an easy and quick assessment to reflect the individual patient's situation in daily life and to document a clinical change. The FOIS can be easily used by physicians and other professional health workers who are not experienced in adult dysphagia management. However, although FOIS is a useful tool to document oral intake, it is also important to pay attention to other characteristics, such as quality of life and nutritional status.

Although only half of our patients remained PEG-tube independent, the majority (three of four patients) had relevant improvement of swallowing (FOIS ⩾ level 3) and, therefore, gained quality of life. The largest case series to date[22] reported that 44% of the patients were able to eat at least soft food; whereas, 56% of their patients needed permanent PEG-tube feeding after a median follow-up of 1.8 years. Other authors reported a clinical success rate without PEG-tube dependency between 30% and 60%[13,23,25,26]. Previous reports found oral intake was possible in 45%-80% of subjects after these procedures[22,23,25,26]. Grooteman *et al*[22] reported that only 24% of the patients were dysphagia-free. We did not investigate dysphagia; rather, we evaluated the diet that was possible during follow-up as this is easy for patients to report on. The findings in literature concerning the dependency on PEG-tube feeding and oral intake are similar to our study.

The most difficult part of the antegrade-retrograde rendezvous procedure is to gain access through the completely obstructed oesophagus. Our data regarding technical success (five of six patients) are in line with the findings of other studies, which reported technical success rates of 83%-100%[13,23,25,26], demonstrating that this procedure is technically feasible. In our series, we started all punctures with guide wires (0.035 inch). It is crucial to puncture with the hard end of the wire, as it is impossible to succeed puncture of a complete oesophageal obstruction with the soft tip of the wire. Furthermore, it is important to reach a good transillumination from the antegradely inserted laryngoscope. The combination of an adequate transillumination and fluoroscopy helps the two operators to bring the two endoscopes as near as

possible and to have the best condition to puncture into the complete obstruction. The endoscopist has to be patient. One can try also other wires as super stiff or Savary wires. One of our cases was a technical failure, after trying to puncture with a super stiff wire, a Savary wire, and argon beamer. In most series[9,13,14,22,26], guidewires were also used. Grooteman *et al*[22] also used electrocautery. Other devices utilized were biopsy forceps[27], needle knifes (*e.g.*, biliary needle knifes)[11,22], and needles, *e.g.*, used in endoscopic ultrasound for fine-needle aspiration[11,14,27]. Schembre *et al*[14] mentioned severe complications, such as abscesses, after using needles and concluded that operators should be very cautious. In a single case, we utilized argon beamer coagulation in addition to the wire and succeeded. Overall, we think that the operator should be aware of the armamentarium of various devices for puncture and should feel confident with the tools utilized.

In our case series, a single case of mediastinal emphysema occurred as a complication of the rendezvous procedure. Fortunately, no surgery was needed. Goguen *et al*[23] reported pneumomediastinum in 18% of subjects, but also oesophageal perforation in 5% and gastrostomy tube site problems in 16% (*e.g.*, leakage by pulling the stomach away from abdominal wall). We only used thin nasal gastroscopes that may have prevented local complications at the gastrostomy tube site; whereas, Goguen *et al*[23] used adult endoscopes. Dellon *et al*[26] also described an oesophageal perforation that could be managed without surgery. However, Zald *et al*[28] reported a fatal venous air embolism after dilation by rendezvous.

One of our patients (a 59-year-old male) developed complete oesophageal obstruction due to toxic epidermal necrolysis (TEN), an idiosyncratic, potentially life-threatening disease characterized by widespread inflammation and necrosis of the epidermis and mucous membranes[29,30]. Mostly caused by a severe adverse event to drugs (*e.g.*, allopurinol or methazolamide)[29], TEN is a very rare disease with an incidence of 0.4-1.2 cases per million person-years. There are very few cases of TEN in the literature, with most encountered in children that resulted in narrowing of the oesophageal lumen through fibrosis and oesophageal obstruction[31]. As far as we know, this is the first report of a rendezvous dilation in a patient suffering from oesophageal complete obstruction caused by TEN.

Several limitations need to be addressed. This is a retrospective study with a small sample size showing a single-centre experience. However, the low number of cases throughout the literature is explained by the rarity of complete obstruction of the hypopharynx/oesophagus. Due to the retrospective nature of this study, we were not able to obtain patients' FOIS levels prior to the endoscopic rendezvous procedure. However, all patients could not swallow at all prior to the procedure, and this correlates to FOIS level 1. Furthermore, details regarding subsequent dilations after rendezvous procedure were not assessed. However, the time interval between and number of dilations over time varied relevantly, based on individual symptoms.

In conclusion, endoscopic antegrade-retrograde rendezvous dilation offers a safe and viable alternative to high-risk blind antegrade dilation of the proximal oesophagus and hypopharynx or surgical approaches in patients with complete proximal oesophageal obstruction. Although only half of the patients remained PEG-tube independent, the majority of patients improved their ability to swallow. We suggest using scores like the FOIS for standardised clinical follow-up.

COMMENTS

Background

Complete oesophageal obstruction is rare and may occur after radiotherapy of head and neck cancers. As antegrade dilation is often unsuccessful, retrograde endoscopic rendezvous dilation can be used to restore oesophageal patency.

Research frontiers

This special endoscopic technique is rarely used. Therefore, it is important to analyse its technical and clinical success.

Innovations and breakthroughs

This case series focuses on the clinical outcome of endoscopic retrograde-antegrade rendezvous dilation.

Applications

This case series showed a fairly good success rate and noticeably few complications. We contributed further information to the existing literature.

Terminology

Gastroscopes are instruments for the examination of the upper gastrointestinal tract. A complete obstruction is a completely occluded lumen. Rendezvous procedure means that one endoscope is used from the proximal part and one from the distal part of the complete obstruction.

Peer-review

This case series provides information about the technical and clinical success of antegrade-retrograde rendezvous dilation of complete oesophageal obstructions, which are rare. This endoscopic technique is interesting for other gastroenterologists.

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ORIGINAL ARTICLE

Observational Study

CD24 genetic variants contribute to overall survival in patients with gastric cancer

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Abstract

AIM: To investigate the role of single nucleotide polymorphisms (SNPs) in *CD24* gene in susceptibility and overall survival of gastric cancer (GC).

METHODS: We genotyped 3 tagging SNPs of *CD24*-P-534 in the promoter region, P170 in the coding region of exon 2 and P1527 in the 3′ untranslated region - using polymerase chain reaction-restriction fragment length polymorphism in specimens from 679 histologically-confirmed GC cases, 111 gastric atrophy (GA) cases and 976 tumor-free controls. Serum

immunoglobulin G antibodies to *Helicobacter pylori* (*H. pylori*) of all subjects were detected by enzyme-linked immunosorbent assay. CD24 expression was evaluated by immunohistochemistry in 131 GC specimens. Correlations between SNPs and risk of GC or GA were shown by *P* values and odd ratios (ORs) with 95% confidence intervals (95%CI) compared with the most common genotype of each SNP using the unconditional logistic regression model after adjusting for age, sex and *H. pylori* infection. Survival within each SNP group was plotted by Kaplan-Meier method and compared by log-rank test (recessive model). Hazard ratios with 95%CIs were computed by Cox regression model after adjusting for age, sex, histological type, tumor differentiation, clinical stage and post-operational chemotherapy.

RESULTS: All of the three loci were in Hardy-Weinberg equilibrium in the control group. Median follow-up time for the 600 GC patients included in the survival analysis was 36.2 mo (range, 2.1-66.7 mo; 95%CI: 34.3-36.5 mo). Patients with the P-534 A/A genotype had significantly shorter survival (HR = 1.38, 95%CI: 1.01-1.88, *P* = 0.042) than did the C/C or C/A genotype carriers after adjusting for age, sex, histological type, tumor differentiation, clinical stage and post-operational chemotherapy. This trend was more evident in patients who lived longer than 2.5 years (HR = 7.55, 95%CI: 2.16-26.32, *P* = 0.001). The P170 T/T genotype was associated with a shorter lifespan than the non-T/T genotypes, but not significantly so. None of the three genetic variants was found to be associated with risk of GC (including tumor stage, grade and distant metastasis) or with risk of gastric atrophy. Furthermore, no difference of CD24 expression was found among the genotypes.

CONCLUSION: The P-534 site in *CD24* gene affects the overall survival of gastric cancer and may serve as a prognostic marker for gastric cancer.

Key words: Gastric cancer; *CD24*; Single nucleotide polymorphisms; Gastric atrophy; Overall survival

Core tip: We evaluated the role of three genetic variants of *CD24* in gastric cancer (GC) risk and prognosis using 679 GC cases and 976 controls. We observed that GC cases with the A/A genotype of P-534 (which lies in the *CD24* promoter) had a significantly shorter survival (HR = 1.38) especially among patients who lived longer than 2.5 years (HR = 7.55) after adjusting for age, sex, histological type, tumor differentiation, clinical stage and post-operational chemotherapy. Our study provides the first evidence that P-534 site in *CD24* may serve as a prognostic marker for gastric cancer.

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies and the third cause of cancer-related death worldwide[1]. *Helicobacter pylori* (*H. pylori*) infection has been established to cause GC and was classified as carcinogenic to humans (Group 1) by IARC in 1994[2]. Although over half of the world's population are estimated to be infected with *H. pylori*[3], relatively few develop GC, and gastric damages induced by *H. pylori* infection vary widely, which together imply a role for host genetic factors in response to chronic *H. pylori* infection and subsequent GC development.

CD24 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface glycoprotein with functions in signal transduction and cell adhesion. It is expressed in a large variety of human malignancies. Over-expression of CD24 results in a more aggressive malignant phenotype with greater proliferative and cell migration capabilities, whereas its down-expression shows a less malignant phenotype[4-6]. CD24 is reportedly associated with tumor growth, invasion, metastasis, recurrence and treatment response in various cancers, including breast cancer[7,8], prostate cancer[9], colorectal cancer[10,11], hepatocellular carcinoma[12], esophageal squamous cell carcinoma[13] and GC[14,15]. CD24 is also a potential marker of cancer stem cells, which possess capabilities for tumorigenesis, self-renewal and producing differentiated progeny[16-18].

In GC, CD24 can mediate carcinogenesis and promote GC progression[14,15]. CD24 expression gradually increases in the progression of normal gastric mucosa, non-atrophic chronic gastritis, chronic atrophic gastritis (CAG), CAG with intestinal metaplasia, dysplasia and finally, GC[14]. Mice with normal *CD24* expression show more gastric inflammation, parietal cell atrophy and gland hyperplasia following *Helicobacter felis* infection compared with *CD24*-null mice which have the genetic background of inbred *CD24*-normal mice[19].

Given the important role of CD24 in GC, we tested whether single nucleotide polymorphisms (SNPs) in the *CD24* gene are associated with genetic susceptibility to GC tumor progression and prognosis in a Chinese population.

MATERIALS AND METHODS

Subjects

From July 2008 to December 2012, patients with

Table 1 Primer sequences used for polymerase chain reaction

Primer	Sequence	Length of product	Endonucleases	Bands
P-534F	5′-AGAGATAACCCTGCCCGAG-3′	209 bp	BsrFI	C: 126 bp + 83 bp
P-534R	5′-CCAAGTTTCCTTTGTTTCCC-3′			
outerF	5′-CCACTTGGCATTTTTGAGGCATCT-3′	1882 bp	-	-
outerR	5′-TGTGTCGAGGCAGTTGTAAAAG-3′			
P170F	5′-CTAAAGAGAATGACCTTGGTGGGTTGAG-3′	404 bp	BstXI	T: 275 bp + 129 bp
P170R	5′-GGATTGGGTTTAGAAGATGGGGAAA-3′			
P1527F	5′-GCCAGGGCAATGATGAATGAG-3′	847 bp	BsrI	TG: 645 bp + 202 bp
P1527R	5′-TGTGTCGAGGCAGTTGTAAAAGAT-3′			

histologically diagnosed GC who underwent tumorectomies at the Department of Gastric and Colorectal Surgery of the First Hospital of Jilin University were invited to join this study. Patients with gastric atrophy (GA) and controls with no tumor history were recruited at the Physical Examination Center of the same hospital during the same period. A total of 1766 individuals, 679 GC cases, 111 GA cases and 976 controls signed the informed consent forms and agreed to participate in the study. The study protocol was reviewed and approved by the Ethics Committee of the First Hospital of Jilin University.

Gastric cancer cases were followed-up by telephone calls three months, six months, and one year after each patient's tumorectomy and every one year thereafter until the end of the study or the death of the patients. Cases would not be included in the survival analysis if (1) they were lost to follow-up by the first telephone interview; or (2) they died of surgical complications in the perioperative period. Survival time was defined as the duration from the date of surgery to the date of death if the patients died, or to the date of the last successful interview if the patients were lost to follow-up or alive until the end of the study. Survival time was right-censored except for patients who died of GC.

Treatment information after surgery was also collected during the follow-up period. Post-operational chemotherapy is defined as at least 3 cycles of chemotherapy received after surgery. One third of the GC patients received this type of therapy. The treatment was classified into three regimens: FOLFOX-4 (combination of 5-fluorouracil, leucovorin and oxaliplatin); XELOX (capecitabine and oxaliplatin) and "other" (such as capecitabine or 5-fluorouracil alone).

Genotyping

Blood samples were collected in EDTA tubes and stored at -80 ℃ until DNA extraction. Genomic DNA was isolated following the protocol provided by the manufacturer (Axygen Biosciences, United States).

The full length of the *CD24* gene was first identified by our previous study and was mapped to Chromosome 6q21 by fluorescence *in situ* hybridization (submitted to NCBI database, accession number FJ226006)[20]. Although SNP data on *CD24* was unavailable in HapMap project or dbVar database, we have identified three linkage disequilibrium (LD) blocks that cover the promoter region and exons 1-2 of the *CD24* locus[20]. Haplotype tagging SNPs (tag SNPs) were identified from the three LD blocks with the pairwise $r^2 \geqslant 0.9$ and miner allele frequency > 0.05.

Three tagging SNPs, P-534C/A, P170C/T and P1527TG/del were genotyped to evaluate the association of *CD24* and genetic susceptibility to GC. P-534C/A is located in the promoter region of *CD24* and is 534bp away from the translation-starting site. P170C/T is located in the coding region of exon 2 and its C-to-T transition leads to an alanine to valine substitution at codon 57 of the CD24 protein. P1527TG/del, 1527bp down from the translation-starting site, is located in the 3′ untranslated region.

Genotypes of the selected sites were determined by polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) as described by Li *et al*[21] and our previous study[20] (Primers used are listed in Table 1 and Figure 1 and were synthesized by Takara, Dalian, China). Briefly, the PCR products of P-534 were digested with endonuclease *Bsr*FI overnight at 37 ℃ and the restriction site indicated the presence of the C allele (126 bp and 83 bp) (All restriction endonucleases were bought from New England Biolabs, United States). For P170 and P1527, a nested PCR in which one of the primers for the first PCR mapped to an intron was performed to increase specificity as the exon 2 of *CD24* shows high homology with the intronless pseudogenes in Chromosomes 1, 15 and Y[22]. The second PCRs were amplified independently for P170 and P1527 using the 1000-fold-diluted products of the first PCR as templates (all reagents for PCR were from Tiangen, Beijing, China). These PCR products were then digested overnight with the restriction enzyme *Bst*XI (for P170, 37 ℃) and *Bsr*I (for P1527, 65 ℃). The T allele of P170 produced two fragments, 275 bp and 129 bp; the TG allele of P1527 produced 645 and 202 bp fragments. All products were separated by electrophoresis on 1.5% agarose gels with ethidium bromide staining and scanned on gel imaging system (Gel Doc™ XR+ system, Bio-Rad, United States). Fifty samples were randomly selected to be genotyped by direct sequencing to confirm the validity of PCR-RFLP;

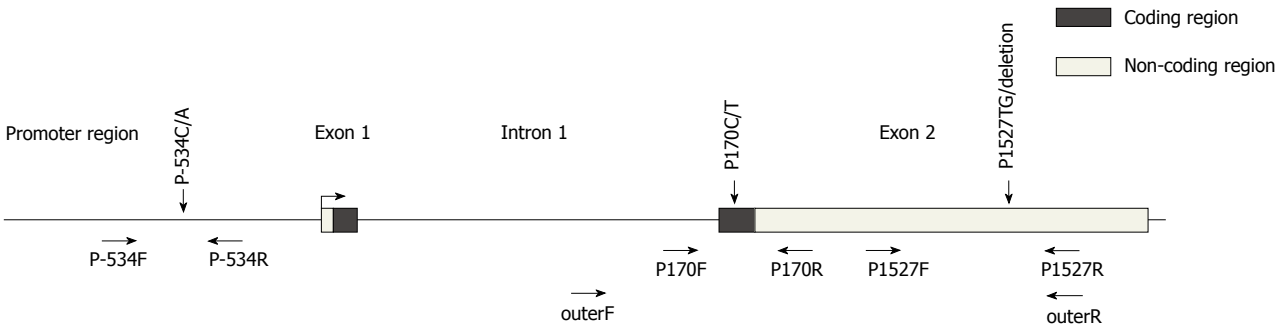

Figure 1 Human *CD24* gene. Primers used were demonstrated by arrows.

three of them with different genotypes were used as positive controls for each PCR-RFLP run. The overall concordance was 100%.

Testing for H. pylori infection and diagnosis of gastric atrophy

Serum immunoglobulin G (IgG) antibodies to *H. pylori* were detected by enzyme-linked immunosorbent assay (ELISA) using *H. pylori*-IgG ELISA kits (Biohit, Finland) according to the manufacturer's protocol. Titers higher than the cut off value of 30 EIU were considered positive for *H. pylori* infection. The inter-day coefficient variations (CV) of the negative and the positive control samples were 4.5% and 1.4%, respectively.

Diagnosis of GA was described elsewhere[23]. Briefly, serum pepsinogen I (PGI) and Ⅱ (PGⅡ) were quantified by ELISA kits (Biohit, Finland). Individuals with PGI < 82.3 ng/mL and PGI/PGⅡ < 6.05 were diagnosed as GA.

Immunohistochemistry of CD24

CD24 expression was assessed in tumor tissue of 131 gastric cancer patients by immunohistochemistry (IHC) method. The detailed procedure is described elsewhere[24]. Briefly, the 4-μm-wide tissue sections were deparaffinized and stained using a streptavidin-biotin immunoperoxidase technique. All slides were then incubated with anti-human CD24 polyclonal antibody (1:100 diluted, sc-7034, Santa Cruz, United States) and developed by 3, 3-diaminobenzidine (DAB). As negative controls, the slides were treated with the IgG isotypes in place of primary antibodies and all negative controls demonstrated negligible background staining. The stained slides were independently evaluated by two pathologists (MSJ and YPW) who were blinded to clinical data and outcomes. The HSCORE system was used to assess the staining results and was calculated by a following equation: HSCORE = ΣPi(i) (I = 0, 1, 2, 3, Pi = 0-100). The i means the intensity of staining (no staining: 0; weak staining: 1; moderate staining: 2; and strong staining: 3). Pi represents percentages (0-100) of stained cells with intensities. The HSCORE ranges from 0 to 300.

Statistical analysis

Continuous data were summarized as medians (25^{th} to 75^{th} percentiles) and compared by Mann-Whitney *U* test or Kruskal-Wallis test. Categorical variables were described as frequencies and percentages and compared using χ^2-test. Correlations between SNPs and risk of GC or GA were demonstrated by *P* values and odd ratios (ORs) with 95% confidence intervals (95%CI) compared with the most common genotype of each SNP. The *P* values and ORs with 95%CIs were calculated using the unconditional logistic regression model after adjusting for age, sex and *H. pylori* infection. Survival functions of the GC patients within each SNP were plotted by Kaplan-Meier method and compared by log-rank test using the recessive model. Hazard ratios (HRs) with 95%CIs were used to quantify the influence of genotypes of each SNP on overall survival and were calculated with Cox regression model after adjusting for age, sex, histological type, tumor differentiation, clinical stage and post-operational chemotherapy. For haplotypes with frequencies > 1%, their associations with risk of GC or GA were assessed compared to the most common haplotype using the logistic regression model with the HAPSTAT software 3.0[25]. Unless otherwise stated, analyses were performed in SAS 9.1.3 software (SAS Institute Inc, United States). A two-tailed *P* value < 0.05 was considered to be statistically significant.

RESULTS

Subject characteristics

A total of 679 GC cases, 111 GA cases and 976 tumor-free controls were included in the study. The baseline characteristics of the subjects are summarized in Table 2. The GC group was oldest in the three group and the control group was youngest (median age: 61.0 years *vs* 50.0 years *vs* 48.5 years, $P < 0.001$ for all pairwise comparisons). And there were more males in the cancer group (71.7% *vs* 59.5% *vs* 59.2%, $P < 0.001$ for comparisons to the atrophy cases or the controls). In the GC group, 67.8% were positive for *H. pylori* infection, significantly higher than the control

Table 2 Characteristics of subjects included *n* (%)

	Cancer	Atrophy	Controls	*P* value
n	679	111	976	
Gender				
Male	487 (71.7)	66 (59.5)	578 (59.2)	< 0.0001
Female	192 (28.3)	45 (40.5)	398 (40.8)	
Age (yr)	61.0	50.0	48.5	< 0.0001
	(54.0-70.0)	(47.0-57.0)	(44.0-55.0)	
≤ 55	204 (30.0)	76 (68.5)	756 (77.5)	< 0.0001
55-70	310 (45.7)	28 (25.2)	181 (18.5)	
> 70	165 (24.3)	7 (6.3)	39 (4.0)	
H. pylori				
Positive	453 (67.8)	84 (75.7)	478 (49.7)	< 0.0001
Negative	215 (32.2)	27 (24.3)	483 (50.3)	
Differentiation				
Poor	437 (68.4)			
Moderate to well	202 (31.6)			
Pathologic type				
Tubular adenocarcinoma	545 (82.2)			
Signet ring cell	50 (7.5)			
Other	68 (10.3)			
TNM stage				
Ⅰ	97 (15.0)			
Ⅱ	227 (35.1)			
Ⅲ	224 (34.6)			
Ⅳ	99 (15.3)			
Distant metastasis				
Positive	573 (85.3)			
Negative	99 (14.7)			
Post-operational Chemotherapy				
No	456 (67.2)			
FOLFOX-4	100 (14.7)			
XELOX	47 (6.9)			
Other	76 (11.2)			

Data are presented as frequency counts (percentage of total) or median (25^{th} to 75^{th} percentiles).

group (49.7%, $P < 0.001$) but non-significantly lower than the GA group (75.7%, $P = 0.097$). Therefore, comparisons of genotype distribution below were adjusted by age, sex and *H. pylori* infection.

The GC cases were mainly of tubular adenocarcinoma type (82.2%), with low-grade differentiation (68.4%), at TNM stage Ⅱ (35.1%) or Ⅲ (34.6%). One third of the cases received chemotherapy after operation (32.8%); 14.7% received FOLFOX-4 and 6.9% received XELOX.

Association of SNPs with risk of gastric cancer or gastric atrophy

All of the three SNPs were in Hardy-Weinberg equilibrium in the control group (P-534: $P = 0.612$; P170: $P = 0.413$; P1527: $P = 0.423$). Distributions of genotypes and alleles are listed in Table 3. Compared with the most common genotype of each SNP, no difference was observed for the distributions of the three loci between the GC and control groups after adjusting for age, sex and *H. pylori* infection. No allele or haplotype was associated with risk of GC. Similar negative results were obtained for GA risk (Table 3). Moreover, no associations were observed between SNPs and risk of *H. pylori* infection (data not shown).

Association of SNPs with clinicopathologic parameters of GC

Genotypic distributions of SNPs were analyzed by clinicopathologic parameters such as histological type, tumor differentiation, TNM stage and distant metastasis in GC cases. However, no significant association was observed (Table 4).

Association of SNPs with survival of gastric cancer

Follow-up information was available for 610 of the 679 GC patients (89.8%). Ten patients died of postoperative complications within 30 d at the beginning of the study period (range, 0-29 d, median: 15.5 d) and these cases were excluded from analyses of effects of SNPs on survival. The median follow-up time for the remaining 600 GC patients was 36.2 mo (range, 2.1-66.7 mo; 95%CI: 34.3-36.5 mo). Two hundred and sixty patients (43.3%) died from GC during the follow-up, 272 patients (45.3%) lived and 68 (11.3%) died of other causes or were lost to follow up.

Survival curves were plotted and compared according to genotypes of each SNP using the recessive model. The patients who carried the A/A genotype of P-534 had shorter survival than those carrying C/C or C/A after adjusting for age, sex, histological type, tumor differentiation, clinical stage and post-operational chemotherapy (HR = 1.38, 95%CI: 1.01-1.88, $P = 0.042$; Table 5 and Figure 2A). This trend was more evident in patients who lived longer than 2.5 years (HR = 7.55, 95%CI: 2.16-26.32, $P = 0.001$). Similarly, the P170 T/T carriers tended to have shorter survival time than the C/C or C/T carriers, although not significantly so (Figure 2B). No meaningful correlation could be observed between the variation of "TG" deletion in P1527 and GC survival ($P = 0.799$).

Multivariate Cox regression analysis showed that three other factors-degree of differentiation, TNM stage and post-operational chemotherapy-were associated with the prognosis of GC. Patient whose GC had low-grade differentiation or advanced clinical stage or who did not received post-operational chemotherapy had shorter survival time (Table 5).

CD24 expressions in tissues of GC

To assess whether SNPs were associated production of CD24 protein, CD24 expression was evaluated in cancerous tissue of 131 GC cases using IHC. Genotypic distributions of the three SNPs in selected cancer cases were similar to non-selected cases (data not shown). CD24 expression was seen mainly in membranes of tumor cells (Figure 3). However, CD24 expression did not observably differ among genotypes of each SNP (Figure 3 and Table 3).

Table 3 Distributions of genotypes in three groups *n* (%)

	Controls	Cancer	*P* value	OR[1] (95%CI)	Atrophy	*P* value	OR[1] (95%CI)
P-534							
C/C	271 (27.8)	181 (26.7)	-	Reference	29 (26.1)	-	Reference
C/A	488 (50.0)	358 (52.7)	0.099	1.1 (0.86-1.48)	62 (55.9)	0.204	1.2 (0.74-1.91)
A/A	217 (22.2)	140 (20.6)	0.145	0.9 (0.61-1.20)	20 (18.0)	0.328	0.8 (0.46-1.54)
C	1030 (52.8)	720 (53.0)	0.886	-	130 (54.1)	0.716	-
A	922 (47.2)	638 (47.0)		1.0 (0.86-1.14)	102 (45.9)		0.9 (0.72-1.25)
P170							
C/C	419 (42.9)	295 (43.4)	-	Reference	60 (54.1)	-	Reference
C/T	439 (45.0)	308 (45.4)	0.277	1.1 (0.82-1.38)	37 (33.3)	0.082	0.6 (0.40-0.96)
T/T	118 (12.1)	76 (11.2)	0.369	0.9 (0.60-1.29)	14 (12.6)	0.724	0.9 (0.47-1.65)
C	1277 (65.4)	898 (66.1)	0.673	-	157 (70.7)	0.114	-
T	675 (34.6)	460 (33.9)		1.0 (0.83-1.12)	65 (29.3)		0.8 (0.58-1.06)
P1527							
TG/TG	826 (84.6)	559 (82.3)	-	Reference	95 (85.6)	-	Reference
TG/del	142 (14.6)	117 (17.2)	0.069	1.3 (0.97-1.82)	15 (13.5)	0.940	0.9 (0.50-1.59)
del/del	8 (0.8)	3 (0.4)	0.162	0.4 (0.09-1.77)	1 (0.9)	0.935	0.9 (0.10-7.30)
TG	1794 (91.9)	1235 (90.9)	0.956	-	205 (92.3)	0.821	-
del	158 (8.1)	123 (9.1)		1.1 (0.88-1.45)	17 (7.7)		0.9 (0.56-1.58)
Haplotype[2]							
ACTG	45.6	47.0	-	Reference	45.4	-	Reference
CTTG	33.6	33.7	0.946	1.0 (0.85-1.16)	28.7	0.407	0.9 (0.63-1.21)
CCTG	11.4	10.2	0.382	0.9 (0.71-1.14)	17.7	0.072	1.6 (0.99-2.37)
CCdel	8.0	9.1	0.370	1.1 (0.87-1.46)	7.7	0.919	1.0 (0.56-1.67)

Data are presented as frequency counts (percentage of total). [1]ORs of the genotypes were calculated adjusting for age, sex and *H. pylori* infection in logistic regression model; [2]The haplotype was lined with P-534, P170 and P1527 and displayed as percentage.

Table 4 Distributions of genotypes according to clinical parameters in gastric cancer cases

	P-534			*P* value	P170			*P* value	P1527		*P* value
	C/C	C/A	A/A		C/C	C/T	T/T		TG/TG	TG/del	
n	181	358	140		295	308	76		559	120	
Age	60 (53-70)	61 (54-71)	61 (55-70)	0.715	61 (53-71)	61 (54-70)	63 (56-70)	0.658	61 (54-70)	60 (51-71)	0.307
Sex											
Male	27.3	50.3	22.4	0.092	43.9	44.2	11.9	0.485	83.6	16.4	0.172
Female	25.0	58.9	16.1		42.2	48.4	9.4		79.2	20.8	
H. pylori											
Positive	26.3	53.4	20.3	0.905	42.6	46.1	11.3	0.769	82.3	17.7	0.996
Negative	27.0	51.6	21.4		45.6	43.7	10.7		82.3	17.7	
Differentiation											
Poor	24.7	55.8	19.5	0.070	43.5	47.4	9.2	0.055	81.5	18.5	0.500
Moderate to well	30.2	46.0	23.8		45.0	40.1	14.8		83.7	16.3	
TNM stage											
Ⅰ-Ⅱ	26.2	52.8	21.0	0.999	45.1	44.7	10.2	0.695	82.4	17.6	0.986
Ⅲ-Ⅳ	26.3	52.6	21.1		42.7	45.2	12.1		82.4	17.6	
Pathologic type											
Tubular adenocarcinoma	26.4	52.3	21.3	0.754	45.0	43.9	11.2	0.173	82.8	17.2	0.751
Signet ring cell	26.0	60.0	14.0		34.0	60.0	6.0		80.0	20.0	
Other	27.9	50.0	22.1		38.2	47.1	14.7		85.3	14.7	
Distant metastasis											
Positive	32.3	48.5	19.2	0.323	40.4	44.4	15.1	0.374	79.8	20.2	0.454
Negative	25.1	53.9	20.9		44.3	45.2	10.5		82.9	17.1	
CD24 staining	60 (0-120)	60 (0-120)	100 (20-140)	0.215	60 (0-120)	60 (20-120)	40 (0-100)	0.482	60 (0-120)	60 (40-100)	0.922

Data are presented as percentage of total or median (25th to 75th percentiles).

Table 5 Results of multivariate Cox regression analysis

	P value	HR	95%CI
P-534			
C/C + C/A	-	1.00	-
A/A	0.0416	1.38	1.01-1.88
Age	0.1875	1.01	1.00-1.02
Sex			
Male	-	1.00	-
Female	0.2315	0.83	0.61-1.13
Differentiation			
Moderate to well	-	1.00	-
Poor	0.0089	1.50	1.11-2.04
Pathologic type			
Tubular adenocarcinoma	-	1.00	-
Signet ring cell	0.4750	0.83	0.50-1.39
Other	0.9469	1.01	0.67-1.53
TNM stage			
Ⅰ	-	1.00	-
Ⅱ	0.0003	5.50	2.18-13.86
Ⅲ	< 0.0001	22.29	9.05-54.89
Ⅳ	< 0.0001	32.12	12.63-81.70
Chemotherapy			
No	-	1.00	-
FOLFOX-4	0.0195	0.64	0.45-0.93
XELOX	0.0021	0.43	0.25-0.74
Other	0.0004	0.45	0.29-0.70

DISCUSSION

In this study, we explored the association between variants of *CD24* gene and GC. We found that patients who harbored the P-534 A/A genotype tended to have shorter survival than those who carry P-534 non-A/A genotypes.

This is the first study on the association between SNPs of *CD24* gene and GC, as no *CD24* SNPs were included in any genome-wide association studies of GC[26,27]. Distribution of the P-534 genotypes of *CD24* differs slightly from that of Caucasian populations. The minor allele C of P-534 in Caucasian population (37.2%[20]) was the major allele (52.8%) in Han Chinese in our study. Distributions of P170 and P1527 were similar to those of other ethnicities[28,29].

Numerous studies have reported that SNPs of *CD24* gene are correlated with risk of various autoimmune diseases, such as systemic lupus erythematosus (SLE)[28-31], multiple sclerosis[28,32-35] and inflammatory bowel disease[20,36]. Li *et al*[21] reported that P170 and P1527 of *CD24* affected risk and progression of chronic hepatitis B infection and Sheng *et al*[37] showed that the T/T genotype of P170 correlated with a 2.96-fold increased of risk of hepatocellular carcinoma. In our study, however, we did not observe any influence of *CD24* polymorphisms on risk of *H. pylori* infection (data not shown, but available on request), gastric atrophy, precancerous lesions of GC that are induced partly by *H. pylori* infection[38,39], or GC (Table 3).

Polymorphisms of CD24 have been related to prognosis in several cancers. In breast cancer, CD24 expression was associated with adverse prognosis[7,8] and *CD24* P170 polymorphism could predict response

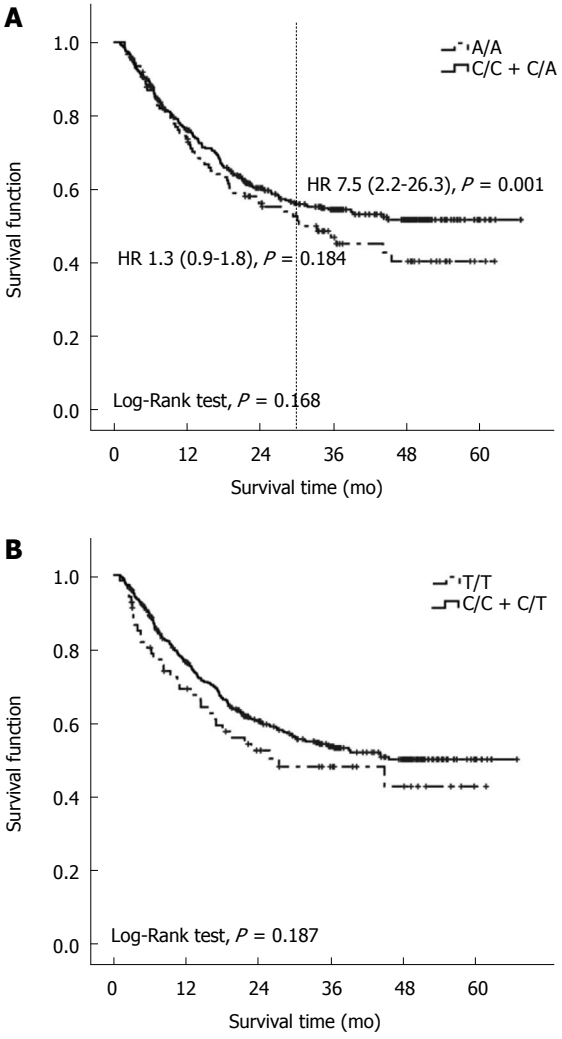

Figure 2 Survival plots of gastric cancer patients. A: Plot for P-534 using the recessive model (A/A *vs* C/C + C/A); B: Plot for P170 using the recessive model (T/T *vs* C/C + C/T).

to chemotherapy[40-42]. In esophageal cancer, P170 of *CD24* was involved in regional lymph node metastasis[43]. In our study, we found that P-534 of *CD24* affected long-term survival of GC, as P-534 A/A genotype carriers have a 7.5-fold increased mortality risk compared wirh non-A/A carriers among patients who lived longer than 2.5 years (Figure 2A). P170 T/T carriers also tended to have shorter survival than did non-T/T carriers, although not significantly so (Figure 2B).

The P-534, which is located in the promoter region of CD24, may influence transcriptional activity. Our previous work showed that a hypomorphic haplotype that contained the C allele of P-534 was associated with risk of multiple sclerosis and this haplotype was involved in higher transcriptional activity and increased expression of CD24 in peripheral blood lymphocytes[20]. The non-synonymous variant P170 may alter the quantity and quality of CD24. Zhou *et al*[32] found that the P170T/T genotype expressed more cell-surface CD24 than did the C/T or C/C genotypes using flow

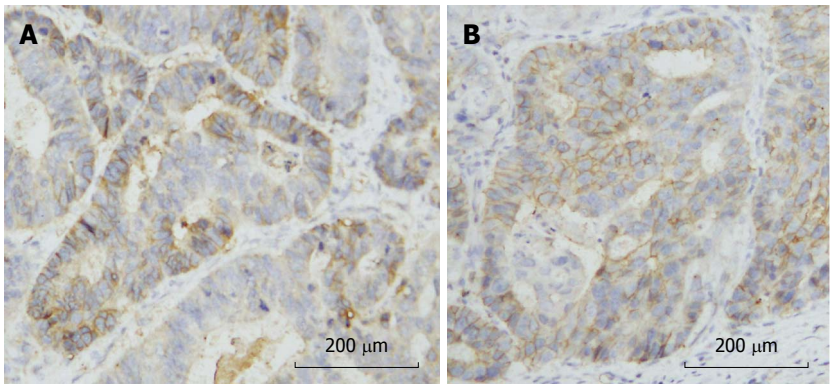

Figure 3 Expression of CD24 by immunohistochemistry. A: expression of CD24 from a gastric cancer case with P-534 AA, P170 CC and P1527 TG/TG genotypes; B: expression of CD24 from a gastric cancer case with P-534 CC, P170 TT and P1527 TG/TG genotypes.

cytometry, which showed an increased risk and more rapid progression of multiple sclerosis. In our study, we evaluated CD24 expression using IHC and found that CD24 was expressed mainly in the membranes of GC cells. However, we did not observe any differences of CD24 expression among genotypes of P-534, P170 or P1527.

Three limitations should be noted in our study. The first one is that some baseline characteristics such as age are different among the three study groups, and we cannot rule out the possibility that individuals in the control group could develop GC when they are older. However, we control the potential influences to the greatest extent by adjusting for these factors in the multivariate analysis. The second is that the follow-up time of the GC cases seems insufficient because most of cases on the right side of the survival plots are censored. This may explain that although individuals carrying P-534 A/A genotype tended to have shorter observable survival time, *P* value from log-rank tests is not significant. However, when we adjust the potential influencing factors and divide the patients to subgroups who live shorter than 2.5 years and those who live longer than 2.5 years, the association of P-534 with long-term survival is statistically significant (HR = 7.5, 95%CI: 2.2-26.3). Nonetheless, longer follow-up time is needed to re-evaluate the role of *CD24* SNPs in prognosis of GC. The last one is that we semi-quantified CD24 expression in tissue using IHC and the influence of SNPs on CD24 might be offset, as protein production can be regulated by factors known and unknown *e.g.*, regulations of transcription, post-transcription and translation. Therefore, more rigorous design should be applied in our future study.

In summary, we find that polymorphisms of the *CD24* gene affect the overall survival of GC, as patients who bear the P-534 A/A genotype tend to have shorter survival than do patients with P-534 non-A/A genotypes. However, we do not observe any associations between *CD24* SNPs and risk of *H. pylori* infection, GA or GC. More studies with larger samples and longer follow-up time are needed to clarify the role of CD24 in GC.

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COMMENTS

Background

CD24 expression as a potential biomarker is associated with poor prognosis in patients with gastric cancer (GC) but its genetic basis still remains to be elucidated.

Research frontiers

GC is one of the most common malignancies and the third cause of cancer-related death worldwide. CD24 mediates gastric carcinogenesis and promotes GC progression. Elucidation of the association between CD24 genetic variants and risk and prognosis of GC may provide novel biomarkers to discriminate individuals with higher risk of GC or to predict prognosis for GC cases.

Innovations and breakthroughs

This study for the first time evaluates the effect of CD24 genetic variations in GC carcinogenesis and prognosis. Its results indicate that CD24 variants may serve as a marker for GC prognosis, but not for carcinogenesis.

Applications

P-534 site of *CD24* might be used as a prognostic predictive marker for GC.

Terminology

CD24 is a glycosylphosphatidylinositol-anchored cell-surface glycoprotein with functions in signal transduction and cell adhesion. CD24 over-expression is associated with a more aggressive malignant phenotype of greater proliferative and migration capability; down-expression shows a less malignant phenotype.

Peer-review

This study investigated the role of *CD24* genetic variants in susceptibility and overall survival of GC and shows that P-534 of *CD24* may serve as an independent prognostic marker for GC.

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CASE REPORT

Rare type of pancreatitis as the first presentation of anti-neutrophil cytoplasmic antibody-related vasculitis

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Abstract

A pancreatic tumor was suspected on the abdominal ultrasound of a 72-year-old man. Abdominal computed tomography showed pancreatic enlargement as well as a diffuse, poorly enhanced area in the pancreas; endoscopic ultrasound-guided fine needle aspiration biopsy and endoscopic retrograde cholangiopancreatography failed to provide a definitive diagnosis. Based on the trend of improvement of the pancreatic enlargement, the treatment plan involved follow-up examinations. Later, he was hospitalized with an alveolar hemorrhage and rapidly progressive glomerulonephritis; he tested positive for myeloperoxidase-anti-neutrophil cytoplasmic antibody (ANCA) and was diagnosed with ANCA-related vasculitis, specifically microscopic polyangiitis. It appears that factors such as thrombus formation caused by the vasculitis in the early stages of ANCA-related vasculitis cause abnormal distribution of the pancreatic blood flow, resulting in non-uniform pancreatitis. Pancreatic lesions in ANCA-related vasculitis are very rare. Only a few cases have been reported previously. Therefore, we report our case and a review of the literature.

Key words: Pancreas; Pancreatitis; Antibodies; Anti-neutrophil cytoplasmic; Vasculitis; Microscopic poly-

angiitis

Core tip: Pancreatic lesions in anti-neutrophil cytoplasmic antibody (ANCA)-related vasculitis are very rare. Only few cases of pancreatic lesions in ANCA-related vasculitis have been reported previously. We encountered a case presenting with pancreatic enlargement and a diffuse, poorly enhanced area in the pancreas during the early stages of ANCA-related vasculitis. In light of the clinical course, it appears that factors such as thrombus formation caused by the vasculitis during the early stages of ANCA-related vasculitis cause abnormal distribution of pancreatic blood flow, resulting in non-uniform pancreatitis manifested in the imaging findings.

Iida T, Adachi T, Tabeya T, Nakagaki S, Yabana T, Goto A, Kondo Y, Kasai K. Rare type of pancreatitis as the first presentation of anti-neutrophil cytoplasmic antibody-related vasculitis. *World J Gastroenterol* 2016; 22(7): 2383-2390 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2383.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2383

INTRODUCTION

Pancreatic lesions in anti-neutrophil cytoplasmic antibody (ANCA)-related vasculitis are very rare. We encountered a patient presenting with pancreatic enlargement and a diffuse, poorly enhanced area in the pancreas during the early stages of ANCA-related vasculitis. Although it is difficult to histopathologically prove findings of vasculitis in the pancreas with endoscopic ultrasound-guided fine needle aspiration biopsy (EUS-FNA) or endoscopic retrograde pancreatography (ERP), in light of the clinical course, it appears that factors such as thrombus formation caused by the vasculitis in the early stages of ANCA-related vasculitis cause to be abnormal distribution of pancreatic blood flow, resulting in non-uniform pancreatitis manifested in the imaging findings described herein. Our report also includes a discussion of the related literature, since there are few previous reports on patients with pancreatic lesions in ANCA-related vasculitis presenting with pancreatitis or nodular shadows in the pancreas.

CASE REPORT

A 72-year-old man had been regularly visiting a clinic for diabetes exhibited weight loss and exacerbation of his diabetes; a pancreatic tumor was suspected on a subsequent abdominal ultrasound, and he was referred to our department in April 2014.

Upon admission, his height and weight were 175 cm and 63.7 kg, respectively; the following were also measured: blood pressure, 130/80 mmHg; pulse, 80 beats/min (regular); and body temperature, 37.0 ℃. He was in a lucid state of consciousness with no neurological abnormalities. There was no anemia in the palpebral conjunctiva or yellowing of the bulbar conjunctiva. Superficial lymph nodes were not palpated. Lung and heart sounds were free of abnormal findings. The abdomen was flat, soft, and without tenderness. There was no lower leg edema.

The laboratory findings upon admission indicated that amylase and lipase were normal (59 IU/L and 53 IU/L, respectively), while trypsin level was slightly elevated (723 ng/mL; normal, 100-500 ng/mL). Findings also indicated mild inflammation: white blood cell count, 10100/μL and C-reactive protein level, 0.97 mg/dL. IgG4 level was normal, and the level of antinuclear antibodies increased 80-fold; carcinoembryonic antigen and cancer antigen 19-9 levels were normal at 2.6 ng/mL and 3.6 U/mL, respectively. Hemoglobin A1c was somewhat elevated at 10.8%.

On chest radiography, a reticular shadow from both hilar areas to the lower lung field was observed.

On abdominal ultrasonography, there was mild swelling from the pancreatic body to the tail, with irregular hypoechoic masses observed in both the margins and interior of the same sites. The splenic artery had traveled through the inside of the tumor; however, the boundary was clear, and there was no obvious invasion into the surrounding adipose tissue. In the pancreatic body, the pancreatic duct was disrupted, but there was no dilation of the cephalic main pancreatic duct.

Contrast-enhanced abdominal/pelvic computed tomography (CT) (Figure 1A-C) showed pancreatic enlargement with a diffuse, poorly enhanced area in the uncinate process and pancreatic body tail.

On magnetic resonance cholangiopancreatography, the path of the main pancreatic duct in the body tail could not be identified, but dilation of the cephalic main pancreatic duct was not observed. Diffusion-weighted images showed diffuse signal changes in the pancreatic body tail.

On EUS (Figure 2), a hypoechoic mass was noted in a form in which the pancreatic lobe structure was maintained in the uncinate process and from the pancreatic body to the tail.

On EUS-FNA, the hypoechoic masses of the uncinate process and pancreatic tail were each punctured three times with a 22G puncture needle (ExpectTM, Boston Scientific, Tokyo, Japan); malignant cells were not detected, and the diagnosis was nonspecific pancreatitis.

On ERP (Figure 3A-C), a slight disparity of the opening diameter was noted in the main pancreatic duct, but localized stenosis or diffuse narrowing were not observed. No abnormality was observed in the

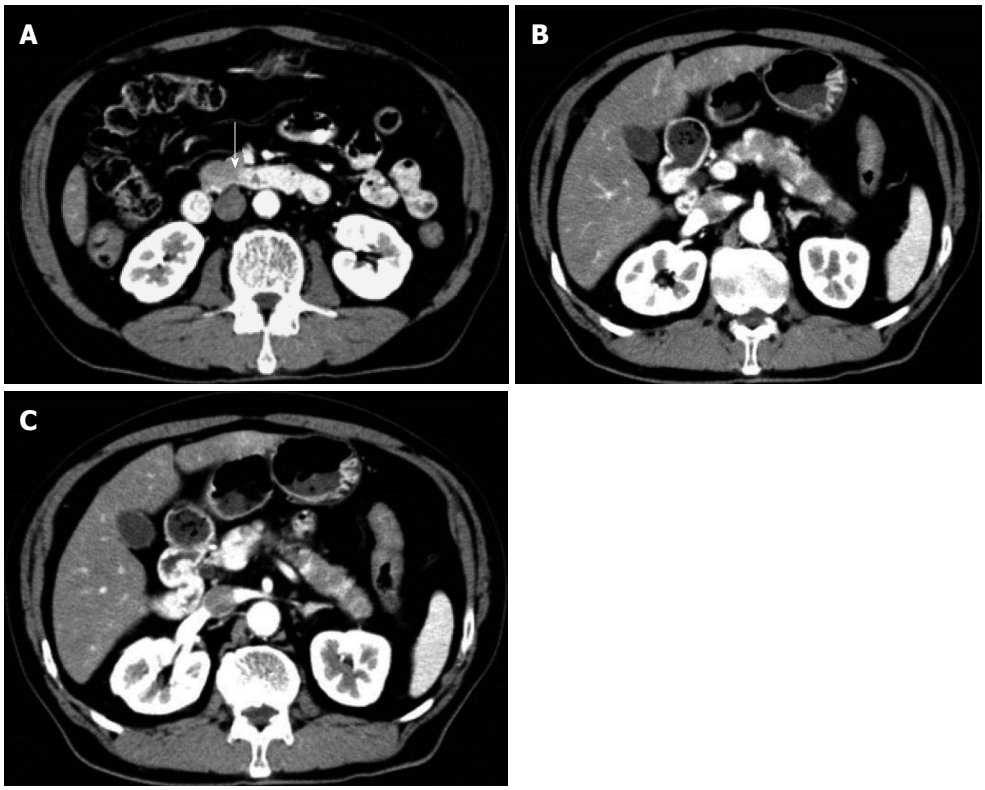

Figure 1 Contrast-enhanced abdominal/pelvic computed tomography showing pancreatic enlargement with a diffuse, poorly enhanced area in the uncinate process (arrow) and pancreatic body tail (A-C).

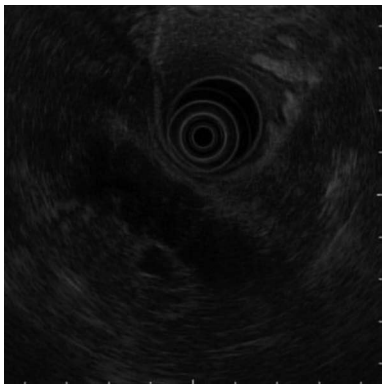

Figure 2 On endoscopic ultrasound, a hypoechoic mass in a form in which the pancreatic lobe structure was maintained in the uncinate process and from the pancreatic body to the tail.

papilla of Vater, but the biopsy revealed thrombus formation.

The patient was suspected of having pancreatic cancer presenting with a special distribution, malignant lymphoma, tumor-forming pancreatitis, or autoimmune pancreatitis (AIP); however, despite the various laboratory tests, a definitive diagnosis was not reached. A contrast-enhanced CT performed in May 2014 showed improvement in the pancreatic enlargement (Figure 4); therefore, it was believed to be nonspecific pancreatitis, and follow-up observation was the chosen strategy. Blood collection and CT scans were subsequently performed every three months, but these did not demonstrate any major changes. However, in January 2015, he was admitted to our hospital's respiratory medicine department for an alveolar hemorrhage and pneumonia (Figure 5); in addition to rapidly progressive glomerulonephritis, he tested positive for myeloperoxidase-ANCA, and he was diagnosed with ANCA-related vasculitis, specifically microscopic polyangiitis (MPA). Contrast-enhanced CT performed during the same period showed that the pancreatic enlargement and poorly enhanced area had disappeared. Despite immunosuppressive therapy with steroids, his respiratory condition worsened, and he passed away in April 2015.

DISCUSSION

The Chapel Hill Consensus Conference (CHCC) published in 1994 classifies the ten diseases of primary vasculitis into three categories by affected vessel size (large vessels, medium vessels, and small vessels)[1]. Although this classification has been widely used around the world, nearly 20 years have passed, and as research on the etiology and pathological condition of vasculitis has advanced, issues with the classification have arisen. A new classification and definitions have been developed and published in January 2013 as "CHCC2012"[2], in which four categories of 16 diseases, including secondary vasculitis and others, have been added to the original three categories of large-vessel vasculitis, medium-vessel vasculitis, and small-vessel

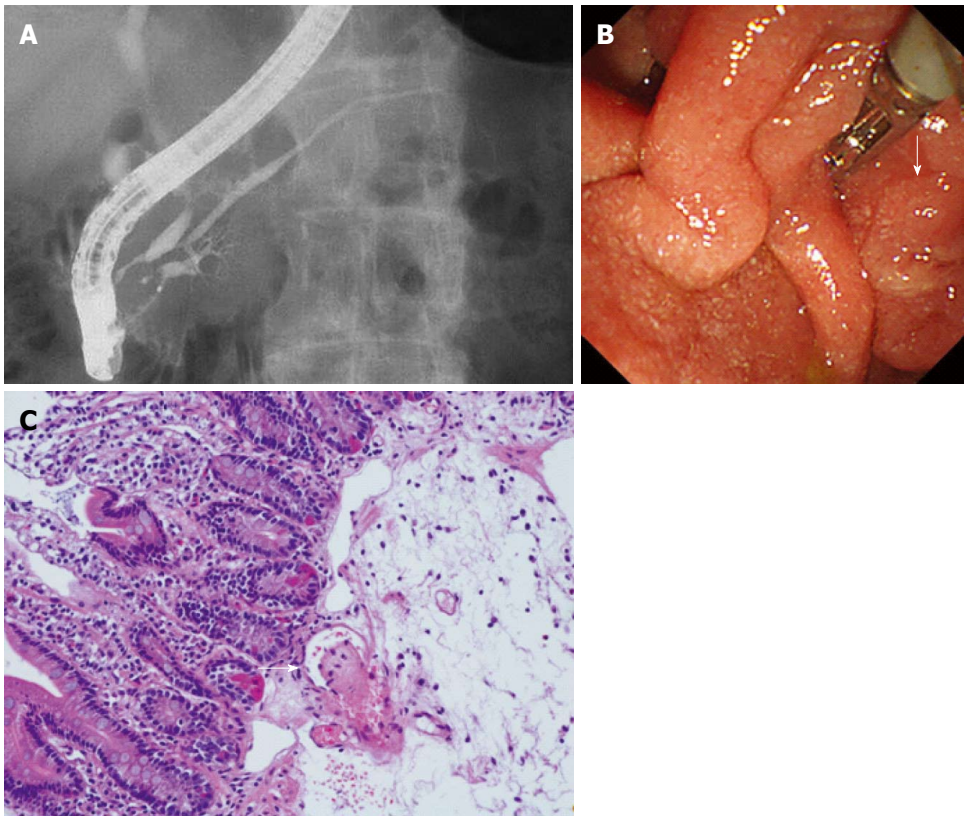

Figure 3 Endoscopic retrograde pancreatography. A: A slight disparity of the opening diameter in the main pancreatic duct, but no localized stenosis or diffuse narrowing observed; B: No abnormality in the papilla of Vater (arrow); C: The biopsy reveals thrombus formation (arrow).

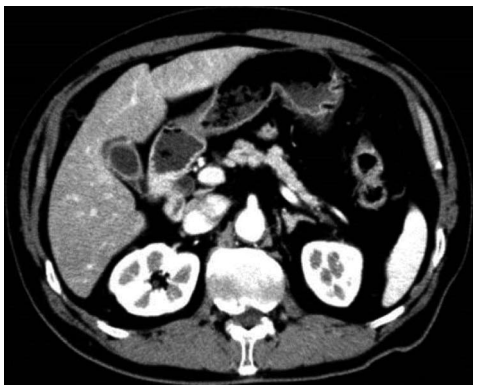

Figure 4 Contrast-enhanced abdominal/pelvic computed tomography performed in May 2014 showing a trend of improvement in the pancreatic enlargement.

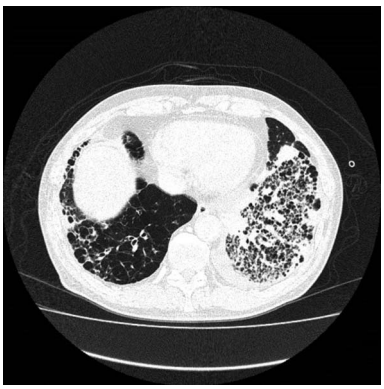

Figure 5 Chest computed tomography demonstrating an alveolar hemorrhage and pneumonia.

vasculitis; the current understanding of vasculitis now encompasses diverse concepts.

In the CHCC2012, MPA belongs to small-vessel vasculitis, with the definition that "MPA is necrotizing vasculitis, with few or no immune deposits, predominantly affecting small vessels (*i.e.*, capillaries, venules, or arterioles). Necrotizing arteritis involving small and medium arteries might be present. Necrotizing glomerulonephritis is very common. Pulmonary capillaritis often occurs. Granulomatous inflammation is absent[2]. The onset of MPA often occurs at ⩾ 50 years of age, and it occurs more frequently in men (male-to-female ratio, 1.5:1)[3]. Sixty percent of cases are positive for myeloperoxidase-ANCA[4], and 51%-94% of cases are reportedly positive for all ANCAs, including PR3-ANCA[4,5]. There are two basic types of findings: those based on bleeding, infarction, and other vascular disorders caused by the rupture of blood vessels and those based on inflammation such as fever and elevated C-reactive protein levels. The main target organs are the kidneys or lungs, with lesions also observed in the skin, muscles, and brain; 30%-50% of lesions are also reportedly observed in the digestive tract[3,6], but it is rare for lesions to be found in the pancreas.

Table 1 Cases presented for pancreatic lesion in anti-neutrophil cytoplasmic antibody-related vasculitis

Authors	Year	Age (yr), sex	Symptoms	Pancreatic enzyme	ANCA	Vasculitis	Pancreatic lesion Imaging	Diagnostic method	Other organ disorder	Treatment Pancreas Other organ	Outcome Pancreas Other organ
O'Neil *et al*[8]	1992	44, M	Jaundice	ND	+	GPA	Ph tumor susp US: hypoechoic CT: 3 cm mass	Renal biopsy	Kidney Nose	Treated PSL, CYC PSL, CYC	Alive Improved Improved
Stuckey *et al*[9]	1992	45, M	Epigastric pain Nausea	AMY: 55	-	GPA	Pancreatitis susp CT: enlargement, sporadic low density lesions	Parotid gland biopsy	Lung	Treated Conservative PSL, CYC	Alive Improved Improved
Berney *et al*[10]	1997	32, M	Epigastric pain	Raised of AMY, Lipase	PR3	MPA	Pancreatitis susp CT: edematous	Renal biopsy	Kidney	Treated Conservative PSL, CYC	Alive Improved spontaneously Improved
Matsubayashi *et al*[11]	2001	65, M	Left abdominal pain	Trypsin: 550 Elastase-I: 440	PR3	GPA	Pbt tumor susp CT: enlargement, sporadic low density lesions	Autopsy	Kidney Lung Spleen	None None None	Died Necrotizing pancreatitis Hemorrhagic pneumonia
Christl *et al*[12]	2004	55, F	Abdominal pain Weight loss	ND	PR3	GPA	Pt tumor susp CT: enlargement, sporadic low density lesions	Postoperative pathology Renal biopsy	Kidney	Treated Ope PSL, CYC	Alive Improved Improved
Iwasa *et al*[13]	2005	84, F	Hematuria Fever	N.D.	MPO	MPA	Normal	Autopsy	Kidney Lung	Treated PSL PSL	Died Necrotizing pancreatitis Lung hemorrhage
Haraguchi *et al*[14]	2005	84, F	Fever	AMY: 130	MPO	MPA	Normal	Autopsy	Kidney Lung	Treated PSL PSL	Died Necrotizing pancreatitis Lung hemorrhage
Tinazzi *et al*[15]	2007	48, F	Epigastric pain	N.D.	-	GPA	Ph tumor susp US: 2cm, mass, hypoechoic MRCP: obstruction of MPD	Postoperative pathology	-	Treated Ope, PSL, CYC, MTX -	Alive Improved -
Joshipura *et al*[16]	2007	47, M	Epigastric pain Fever	AMY: 874 Lipase: 1294	PR3	GPA	Pancreatitis susp CT: Pbt edematous	Turbinate biopsy	Nose	Treated PSL, CYC PSL, CYC	Alive Improved Improved
Abu-Hilal *et al*[17]	2008	20, F	Epigastric pain Nausea	Normal	PR3	GPA	Pancreatitis susp CT: Pt edematous	Renal biopsy	Kidney Lung Skin Intestine	Treated Conservative PSL, CYC	Died Improved spontaneously Lung hemorrhage

Chawla *et al*[18]	2011	60, F	Epigastric pain Nausea	Lipase: 1316	PR3	GPA	Pancreatitis susp CT: diffusely edematous, Ph hypo attenuated lesion	Renal biopsy	Kidney Lung Heart	Treated Conservative PSL, CYC, AZA	Alive Improved spontaneously Improved
Valerieva *et al*[19]	2013	62, F	Epigastric pain	Normal	PR3	GPA	Pt tumor susp CT: Pt, 3cm, mass	Postoperative pathology	-	Treated Ope, PSL, AZA	Alive Improved
Iida *et al* (this case)	2015	72, M	Weight loss	Trypsin: 723	MPO	MPA	Pancreatitis susp CT: enlargement, multiple hypo attenuated lesions	Labo data Imaging Vater biopsy	Kidney Lung	- Treated Conservative PSL, CYC	Died Improved spontaneously Lung hemorrhage

ANCA: Anti-neutrophil cytoplasmic antibody; ND: No data; AMY: Amylase; Ph: Pancreas head; Pbt: Pancreas body and tail; Pt: Pancreas tail; MRCP: Magnetic resonance cholangiopancreatography; MPD: Main pancreatic duct; PSL: Prednisolone; CYC: Cyclophosphamide; Ope: Operation; MTX: Methotrexate AZA: Azathioprine; M: Male; F: Female.

In the present case, it was difficult to histopathologically prove findings of vasculitis in the pancreas with EUS-FNA or endoscopic retrograde cholangiopancreatography; however, lung lesions progressed later, and it is characteristic that imaging showed abnormalities in the pancreas before the diagnosis of ANCA-related vasculitis could be determined. Initially, ANCA was not measured, and the differential diagnoses included pancreatic cancer, malignant lymphoma, autoimmune pancreatitis, and tumor-forming pancreatitis. Irregular, hypoechoic masses were observed in the pancreas, and carcinoembryonic antigen and cancer antigen 19-9 levels were elevated; however, any findings of pancreatic cancer were negated by the fact that no abnormality was observed in the pancreatic ducts and the lesions were scattered (regarding a diagnosis of cancer of the entire pancreas). Malignant lymphoma was considered as a differential disease in part because the artery penetrated the lesion, but enlarged lymph nodes were not observed, and a pathologically definitive diagnosis could not be reached. The diagnosis of AIP was rejected because, although pancreatic enlargement was observed, the diffuse poorly enhanced area was nonspecific, and hyper-IgG4 disease, irregular narrowing of the main pancreatic duct in ERP, or extrapancreatic lesions were not observed. Regarding tumor-forming pancreatitis, tumor signs were not localized, and the pancreatic duct images also showed no abnormalities; therefore, it could not be said to be typical. Although the abdominal pain was not clear, his mild inflammatory reaction, elevated trypsin levels, and pancreatic enlargement suggested pancreatitis. In light of his clinical course, it appears that factors such as thrombus formation caused by the vasculitis in the early stages of ANCA-related vasculitis caused pancreatic blood flow to be abnormally distributed, resulting in non-uniform pancreatitis and manifesting in the presentation of the imaging findings. In AIP, IgG4-positive cells are reportedly detected from the papilla of Vater in a high proportion of biopsies[7]; in the present case, the papilla of Vater was biopsied to exclude AIP even though no abnormalities were observed endoscopically. IgG4-positive cells were not observed, but thrombus formation was present (Figure 3B); conceivably, this also occurred in the peripancreatic vessels, causing an abnormal distribution of the pancreatic blood flow and producing irregular pancreatitis.

When PubMed was searched for previous reports published between 1990 and 2015 of ANCA-related vasculitis presenting with pancreatic lesions, 12 cases were found[8-19] (Table 1). The mean age was 55.2 years (20-84 years), with a male-to-female ratio of 6:7; 11 cases were ANCA-positive, and granulomatosis with polyangiitis was the most frequent diagnosis (9 cases), while there were 4 cases of MPA and no cases of eosinophilic granulomatosis with polyangiitis. In 6 cases, symptoms were accompanied by findings of pancreatitis on imaging; 5 cases presented with nodular shadows that were difficult to differentiate from tumors in the pancreas, and no antemortem pancreatic lesions were indicated, but postmortem autopsy indicated pancreatic lesions in 2 cases. Regarding pathologically definitive diagnoses of pancreatic lesions, 3 cases were diagnosed following a surgical procedure because of the difficulty differentiating the lesions from a tumor, 3 cases were diagnosed by postmortem autopsy, and 7 cases had a diagnosis indirectly proven by biopsies from other organs because a definitive diagnosis could not be reached for the pancreas. None of the cases had a definitive diagnosis of ANCA-related vasculitis based on pancreatic lesions before treatment. Thus, similar to the present case, pancreatic lesions in ANCA-related vasculitis are extremely difficult to diagnose by endoscopic biopsy or similar methods before

treatment, and it is important to indirectly diagnose the condition from the involvement of other organs.

Regarding the treatment of pancreatic lesions, of the 6 cases that presented with findings of pancreatitis on imaging, 5 were successfully treated with conservative treatment. Therefore, it is possible that pancreatitis caused by ANCA-related vasculitis could follow a transient course; however, there are few reports in which ANCA-related vasculitis resolved spontaneously[20], and this remains largely speculative. Despite the possibility that the pancreatitis is transient, remission of pancreatitis has been followed by organ failure in other cases owing to the manifestation of ANCA-related vasculitis in other organs such as the kidneys or lungs, and the patient died, as in the present case. Therefore, we believe that ANCA-related vasculitis should be differentially included as a cause of pancreatitis of unknown etiology, and an early and appropriate diagnosis is required, followed by the start of treatment.

In addition to cases with ANCA-related vasculitis, some cases with Kawasaki disease, polyarteritis nodosa, or other types of vasculitis have presented with pancreatitis or nodules in the pancreas[21,22]. As already described, although the CHCC2012 classifies vasculitis by vascular diameter, there is considerable overlap in the vessels invaded in each group, and vessels of any size can be affected; therefore, we feel that care should be taken regarding the relationship with pancreatic lesions in all forms of vasculitis, including ANCA-related vasculitis. However, because there are so few previous reports regarding vasculitis and pancreatic lesions, the details of the relationship remain unclear, and additional cases will need to be described.

COMMENTS

Case characteristics

Pancreatic lesions in anti-neutrophil cytoplasmic antibody (ANCA)-related vasculitis are very rare. This is a case of rare type of pancreatitis as the first presentation of anti-neutrophil cytoplasmic antibody-related vasculitis.

Clinical diagnosis

Factors such as thrombus formation caused by the vasculitis in the early stages of ANCA-related vasculitis cause abnormal distribution of the pancreatic blood flow, resulting in non-uniform pancreatitis.

Differential diagnosis

The patient was suspected of having pancreatic cancer presenting with a special distribution, malignant lymphoma, tumor-forming pancreatitis, or autoimmune pancreatitis.

Laboratory diagnosis

On second admission, myeloperoxidase-ANCA was positive.

Imaging diagnosis

First, it was believed to be nonspecific pancreatitis because of improvement in the pancreatic enlargement.

Pathological diagnosis

On endoscopic ultrasound-guided fine needle aspiration biopsy, malignant cells were not detected, and the diagnosis was nonspecific pancreatitis. On endoscopic retrograde pancreatography, no abnormality was observed in the papilla of Vater, but the biopsy revealed thrombus formation.

Treatment

First, it was believed to be nonspecific pancreatitis, and computed tomography showed improvement in the pancreatic enlargement, follow-up observation was the chosen strategy. For an alveolar hemorrhage and pneumonia, immunosuppressive therapy with steroids was performed.

Related reports

ANCA-related vasculitis presenting with pancreatic lesions published between 1990 and 2015 were found 12 cases in PubMed.

Experiences and lessons

ANCA-related vasculitis should be differentially included as a cause of pancreatitis of unknown etiology, and an early and appropriate diagnosis is required, followed by the start of treatment.

Peer-review

The case report by Iida T and co-workers describes a rare case of ANCA-related vasculitis with pancreatic involvement. It is a well-constructed case report and a short but good review of the corresponding literature.

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CASE REPORT

Cystic micropapillary neoplasm of peribiliary glands with concomitant perihilar cholangiocarcinoma

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Institutional review board statement: The study was reviewed and approved by the Shizuoka Cancer Center Hospital Institutional review board.

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Abstract

We report a case of a 75-year-old man with cystic micropapillary neoplasm of peribiliary glands detected preoperatively by radiologic examination. Enhanced computed tomography showed a low-density mass 2.2 cm in diameter in the right hepatic hilum and a cystic lesion around the common hepatic duct. Under a diagnosis of perihilar cholangiocarcinoma, right hepatectomy with caudate lobectomy and bile duct resection were performed. Pathological examination revealed perihilar cholangiocarcinoma mainly involving the right hepatic duct. The cystic lesion was multilocular and covered by columnar lining epithelia exhibiting increased proliferative activity and p53 nuclear expression; it also contained foci of micropapillary and glandular proliferation. Therefore, the lesion was diagnosed as a cystic micropapillary neoplasm of peribiliary glands and resembled flat branch-type intraductal papillary mucinous neoplasm of the pancreas. Histological examination showed the lesion was discontinuous with the perihilar cholangiocarcinoma. Immunohistochemistry showed the cystic neoplasm was strongly positive for MUC6 and that the cholangiocarcinoma was strongly positive for MUC5AC and S100P. These results suggest these two lesions have different origins. This case warrants further study on whether this type of neoplasm is associated with concomitant cholangiocarcinoma as observed in pancreatic intraductal papillary mucinous neoplasm with concomitant pancreatic duct adenocarcinoma.

Key words: Intraductal papillary neoplasm of the bile duct; Peribiliary gland; Gastric type; Perihilar

cholangiocarcinoma

Core tip: This report highlights intraductal papillary neoplasm of bile duct (IPNB) arising from peribiliary glands (PBGs) in a case of perihilar cholangiocarcinoma, which is of special interest because to our knowledge this is the first report of IPNB arising from PBGs with concomitant perihilar cholangiocarcinoma occurred separately, and the neoplasm may be regarded as the biliary counterpart of branch duct-type intraductal papillary mucinous neoplasm of pancreas with concomitant pancreatic duct adenocarcinoma.

Uchida T, Yamamoto Y, Ito T, Okamura Y, Sugiura T, Uesaka K, Nakanuma Y. Cystic micropapillary neoplasm of peribiliary glands with concomitant perihilar cholangiocarcinoma. *World J Gastroenterol* 2016; 22(7): 2391-2397 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2391.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2391

INTRODUCTION

Intraductal papillary neoplasm of the bile duct (IPNB) is characterized by exophytic proliferation of neoplastic epithelial cells with fine fibrovascular stalks in the dilated bile duct lumen and mucin hypersecretion[1-3]. IPNB and pancreatic intraductal papillary mucinous neoplasm (IPMN) share some clinicopathological characteristics[1-5], and IPNB can be regarded as a biliary counterpart of IPMN. However, IPNB differs from IPMN in several aspects. In particular, IPMN can be divided into the main pancreatic duct and branch pancreatic duct types[6]. Meanwhile, most previously reported cases of IPNB occurred in the extrahepatic, hilar, and large intrahepatic bile ducts; such IPNB cases can be regarded as counterparts of IPMN, particularly the main duct type[4,6,7]. So far, the IPNB type corresponding to branch duct-type IPMN (BD-IPMN) remains to be established.

Several cases of IPNB arising from peribiliary glands (PBGs) exhibiting grossly visible papillary lesions have recently been reported[8-10]. Nakanuma *et al*[4,5,11] recently reported that the biliary tract can be regarded as an incomplete pancreas and that PBGs and their own conduits around the large bile ducts may correspond to the branches of the pancreatic duct and exocrine pancreas[12]. In this context, IPNBs arising from PBGs could be regarded as branch duct-type IPNB (BD-IPNB) corresponding to BD-IPMN. Sato *et al*[13] recently reported an entity of cystic and micropapillary epithelial lesions of PBGs by surveying many autopsy cases; these lesions are characterized by grossly visible multicystic epithelial tumors with foci of micropapillary patterns. After extensive analysis, they concluded these lesions are neoplastic; therefore, the term "cystic micropapillary neoplasm of PBGs" is preferable in this context.

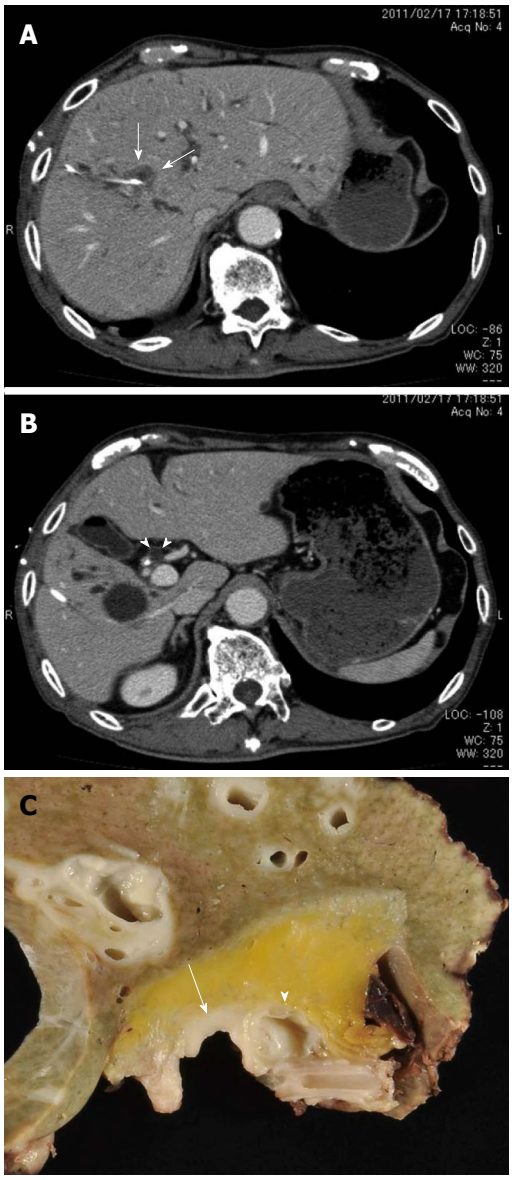

Figure 1 Preoperative images. A: Enhanced computed tomography showing a 2.2-cm low-density tumor (arrows) in the right hepatic hilum. The white tube is the percutaneous transhepatic biliary drainage tube; B: Enhanced computed tomography showing a cystic lesion (arrowheads) by the common bile duct; the endoscopic nasobiliary drainage tube appears as a white dot. A solitary cyst can also be observed in the right lobe; C: Surgically resected specimen showing a cystic lesion (arrowhead) in the hilar connective tissue near the common bile duct involving the carcinoma (arrow).

IPNB is a pre-invasive lesion that is eventually followed by invasive carcinoma (*i.e.*, IPNB with an associated invasive carcinoma)[1,2]. IPMN is also a pre-invasive neoplasm that is followed by invasive carcinoma. In addition, IPMN is not uncommonly associated with concomitant pancreatic duct adenocarcinoma (PDAC) (*i.e.*, IPMN with concomitant PDAC)[14-16], while no such cases of IPNB with concomitant cholangiocarcinoma (CCA) have been reported. Herein, we report a case of cystic micropapillary neoplasm of PBGs that was

detected preoperatively by radiologic examination. The neoplasm was associated with perihilar CCA, suggesting concomitance similar to BD-IPMN with concomitant PDAC.

CASE REPORT

A 75-year-old man complaining of obstructive jaundice was referred to our hospital. He previously underwent endoscopic mucosal dissection for gastric cancer and pharyngolaryngoesophagectomy for hypopharyngeal carcinoma. Laboratory test results on admission were as follows: aspartate aminotransferase, 87 IU/L (normal: 10-40 IU/L); alanine aminotransferase, 84 IU/L (normal: 5-40 IU/L); γ-glutamyl transpeptidase, 309 IU/L (normal: < 70 IU/L); alkaline phosphatase, 909 IU/L (normal: 115-359 IU/L); total bilirubin, 8.6 mg/dL (normal: 0.2-1.0 mg/dL); carcinoembryonic antigen, 2.7 ng/mL (normal: < 5.0 ng/dL); and carbohydrate antigen 19-9, 112 IU/mL (normal: < 37 IU/mL). After hospitalization, serum total bilirubin and liver enzyme levels normalized because of endoscopic nasobiliary drainage and percutaneous transhepatic biliary drainage. Enhanced computed tomography showed a low-density mass 2.2 cm in diameter in the right hepatic hilum (Figure 1A) and a cystic lesion near the common bile duct (Figure 1B); this cyst was retrospectively diagnosed as a peribiliary cyst. After a pathological diagnosis of adenocarcinoma on the basis of cholangioscopic biopsy, right hepatectomy with caudate lobectomy and bile duct resection were performed. The postoperative course was uneventful, and he was discharged on the 17th postoperative day. During follow-up, multiple liver metastases were found, and he died one year postoperatively.

Pathological findings

Macroscopic examination revealed the tumor spread from the right hepatic duct to the common hepatic duct. The cystic lesion was 1.0 cm × 1.0 cm and had a multilocular appearance in the hilar connective tissue near the common bile duct (Figure 1C, 2A and 2B).

Histologically, the tumor was well-to-moderately differentiated tubular adenocarcinoma of the right hepatic duct extending to the common bile duct (Figure 2A and Figure 3) and was therefore diagnosed as perihilar CCA. The cystic lesion was multilocular with fine fibrous septae. A large part of the inner surfaces of cystic spaces were covered with columnar epithelium resembling gastric epithelia (Figure 2C). Glandular components resembling pyloric glands were focally found under the lining epithelia of cystic spaces (Figure 2D). Micropapillary projections with intermediate-grade intraepithelial neoplasia were focally found (Figure 2E). These cystic lesions were rimmed by non-neoplastic PBGs (Figure 2F). In addition, the lesion was near the common hepatic duct, suggesting it might have originated from PBGs. These findings were compatible with a diagnosis of cystic micropapillary neoplasm of PBGs[13]. The hilar CCA and cystic micropapillary neoplasm were close but not continuous (Figure 2A).

Immunohistochemical staining for MUC1, MUC2, MUC5AC, MUC6, CK7, CK20, S100P, estrogen receptor, progesterone receptor, Ki67, and p53 was performed (Figure 4 and Table 1). No ovarian-like stroma or stromal cells positive for estrogen receptor or progesterone receptor were observed, indicating this cystic neoplasm differed from the hepatic mucinous cystic neoplasm. The perihilar CCA was strongly and diffusely positive for MUC5AC and S100p, while the cystic papillary neoplasm was strongly and diffusely positive for MUC6 and focally positive for MUC5AC. Both were strongly positive for CK7 and focally positive for p53. These findings suggest both neoplasms had different phenotypes.

DISCUSSION

There have been several reports of neoplastic changes of PBGs[8-10,17], showing that CCA[17] and grossly visible papillary neoplasms can arise from PBGs[4,5,10]; the latter are termed BD-IPNB[5,8-10]. However, the clinicopathological characteristics of BD-IPNB remain incompletely understood. Sato *et al*[13] recently found 9 cases of cystic micropapillary neoplasms of PBGs among 938 consecutive autopsied livers. In the present case, the lesion comprised multilocular cysts covered by columnar epithelial cells with mucinous cytoplasm, increased proliferative activities, and frequent nuclear expression of p53. In addition, the lesion exhibited micropapillary projections. The lesion was located in the hilar connective tissue near the common bile duct and was rimmed by non-neoplastic PBGs. Hence, this lesion was diagnosed as cystic micropapillary neoplasm of PBGs.

The present case differs from previously reported BD-IPNBs in that papillary lesions were not grossly visible; rather, this case histologically resembled flat BD-IPMN[6,15,16,18]. Furthermore, this lesion was positive for MUC6 and focally positive for MUC5AC, suggestive of gastric-type phenotype and phenotypically similar to flat BD-IPMN[6]. In fact, there were several foci of pyloric glands beneath the cyst-lining surface epithelia within the lesion. In this context, the lesion could be also termed branch-type IPNB without a grossly visible papillary component (*i.e.*, flat-type BD-IPNB), such as flat BD-IPMN.

In the pancreas, IPMN is not infrequently associated with PDAC (*i.e.*, IPMN with concomitant PDAC)[15,16,18]. Retrospective studies on BD-IPMN with concomitant PDAC report its incidence among IPMNs to range from 3.3%-9.2%[15,16,19]. The clinical outcome of patients with IPMN with concomitant PDAC is better than that of ordinary PDAC (*i.e.*, without concomitant IPMN); this is because IPMN concomitant with pancreatic cancer is detected at an earlier stage because of

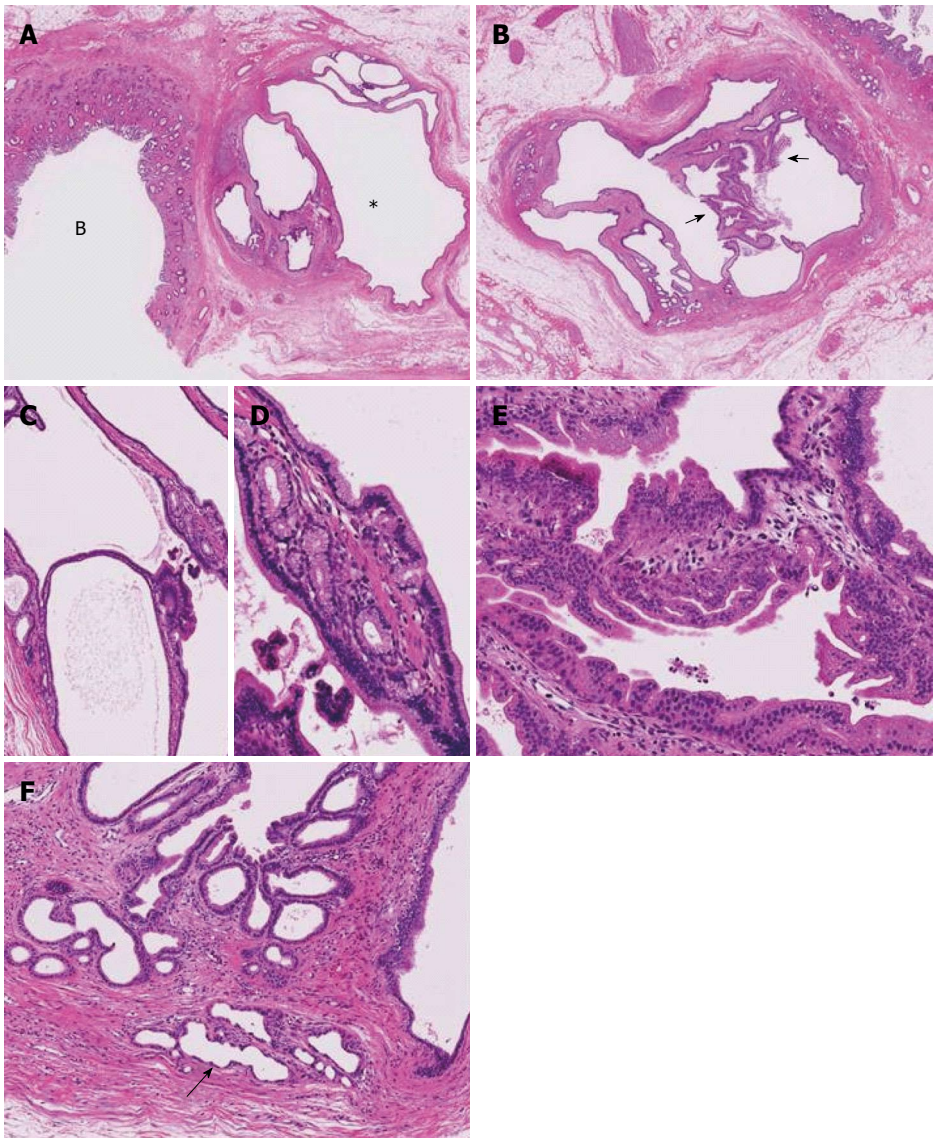

Figure 2 Pathological findings. A: Low-power histologic image of the same region showing a multilocular cystic lesion (*) and the common hepatic duct involving the carcinoma; B: Another level of a cystic lesion shows a multilocular cystic lesion with some areas exhibiting a micropapillary pattern (arrows), HE; C: The multilocular parts show fine septae lined by columnar epithelium, HE; D: The lining epithelium is columnar with clear cytoplasm, and a pyloric glandular pattern is visible underneath, HE; E: Micropapillary patterns, HE; F: The border of the cystic lesion is rimmed by non-neoplastic peribiliary glands (arrow), HE. HE: Hematoxylin and eosin.

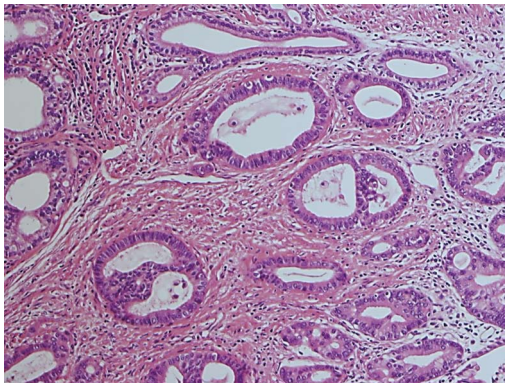

Figure 3 Well-to-moderately differentiated tubular adenocarcinoma of the perihilar bile duct (hematoxylin and eosin).

the presence of IPMN[16]. Gastric-type BD-IPMN with concomitant PDAC does not exhibit GNAS mutation in contrast to the majority of gastric-type IPMNs[18].

Interestingly, the lesion in the present case was associated with perihilar CCA; they were discontinuous, and their phenotypes were different, suggesting the flat BD-IPNB and hilar CCA occurred separately. Such an association has not been reported in cases of hilar CCA or IPNB; furthermore, our survey of 59 surgically resected perihilar CCAs at our hospital failed to find another case of cystic micropapillary neoplasm of PBGs (*i.e.*, flat BD-IPNB). Therefore, to our knowledge the case presented herein could be the first case of flat BD-IPNB with concomitant perihilar CCA.

Because few cases of flat BD-IPNB have been

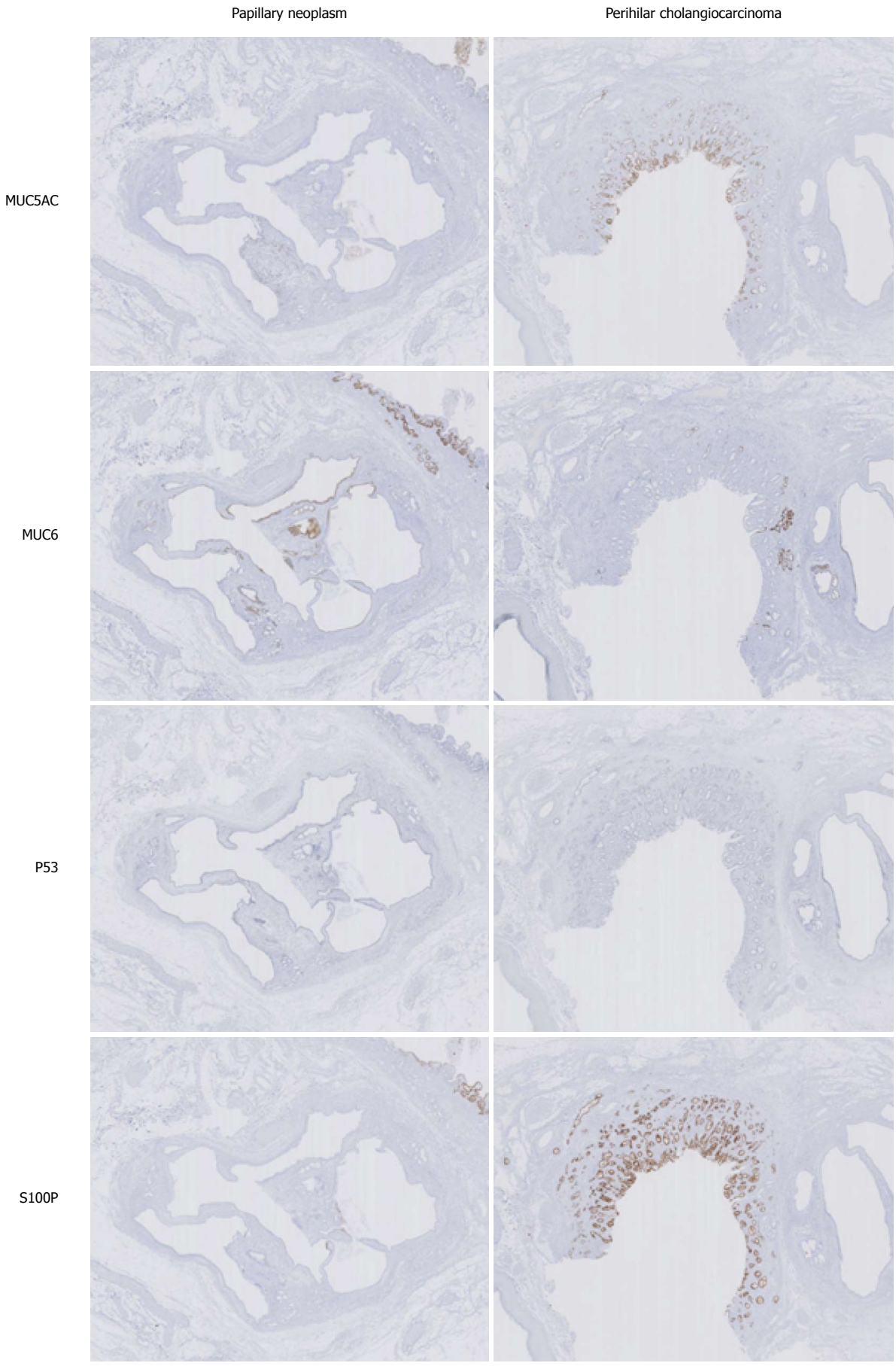

Figure 4 Immunohistochemistry of papillary neoplasm and Perihilar cholangiocarcinoma.

Table 1 Immunohistochemical profiles of papillary neoplasm and perihilar cholangiocarcinoma

	Papillary neoplasm	Perihilar cholangiocarcinoma
MUC1	+	++
MUC2	-	-
MUC5AC	+	+++
MUC6	+++	-
p53	+	+
Ki67	20%	30%
CK7	+++	+++
CK20	-	-
S100P	-	++
ER	-	-
PgR	-	-

reported, it is unknown if flat BD-IPNB is associated with a high incidence of CCA. Therefore, the recognition of flat BD-IPNB or cystic micropapillary neoplasm of PBGs as well as analyses of more cases of flat BD-IPNB with or without concomitant CCA are required.

In conclusion, this is the first report of cystic micropapillary neoplasm of PBGs (*i.e.*, flat type BD-IPNB) resembling flat BD-IPMN with concomitant perihilar CCA to our knowledge. Further studies are required to establish the concept of flat BD-IPNB with concomitant perihilar CCA.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A 75-year-old man complained of obstructive jaundice.

Clinical diagnosis

His skin and palpebral conjunctiva showed jaundice.

Differential diagnosis

Malignant tumors (cholangiocarcinoma, pancreatic cancer, carcinoma of the ampulla of Vater) and benign lesions (common bile duct stone, cholangitis).

Laboratory diagnosis

He had elevated hematological values for aspartate aminotransferase (87 IU/L) alanine aminotransferase (84 IU/L), γ-glutamyl transpeptidase (309 IU/L), alkaline phosphatase (909 IU/L), total bilirubin (8.6 mg/dL), carcinoembryonic antigen (2.7 ng/mL) and carbohydrate antigen 19-9 (112 IU/mL).

Imaging diagnosis

Enhanced computed tomography showed a low-density mass 2.2 cm in diameter in the right hepatic hilum and a cystic lesion near the common bile duct.

Pathological diagnosis

Intraductal papillary neoplasm of bile duct (IPNB) arising from peribiliary glands (PBGs) with concomitant perihilar cholangiocarcinoma occurred separately.

Treatment

Right hepatectomy with caudate lobectomy and bile duct resection were performed.

Term explanation

IPNB is characterized by exophytic proliferation of neoplastic epithelial cells with fine fibrovascular stalks in the dilated bile duct lumen and mucin hypersecretion.

Experiences and lessons

This is the first report of cystic micropapillary neoplasm of PBGs resembling flat branch duct-type (BD)-intraductal papillary mucinous neoplasm (IPMN) with concomitant perihilar concomitant cholangiocarcinoma (CCA) to our knowledge and further studies are required to establish the concept of flat BD-IPNB with concomitant perihilar CCA.

Peer-review

This is a case report presenting a cystic micropapillary neoplasm of peribiliary glands with concomitant perihilar cholangiocarcinoma. The authors conclude that this case warrants further study as it may be correspond to the combination of IPMN with concomitant pancreatic duct adenocarcinoma. This is the first case of such combination of tumours and deserves publication.

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CASE REPORT

Laparoscopic resection of adult colon duplication causing intussusception

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Author contributions: Kyo K served as an attending doctor for the patient, designed the report and wrote the manuscript; Kyo K and Azuma M performed the surgery; Azuma M, Okamoto K, Nishiyama M, Shimamura T, Maema A, Shirakawa M, Nakamura T, Koda K and Yokoyama H were involved in editing the manuscript; Koda K performed the pathological examination.

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Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of Fujieda Municipal General Hospital.

Informed consent statement: Written informed consent was obtained from the patient for publication of this case report and the accompanying images.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest for this article.

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Abstract

Gastrointestinal duplications are uncommon congenital malformations that can occur anywhere along the gastrointestinal tract. Most cases are recognized before the age of 2 years, and those encountered in adults are rare. We describe here a case of ascending colon duplication in a 20-year-old male that caused intussusception and was treated laparoscopically. Although computed tomography revealed a cystic mass filled with stool-like material, the preoperative diagnosis was a submucosal tumor of the ascending colon. We performed a laparoscopic right colectomy, and the postoperative pathological diagnosis was duplication of the ascending colon, both cystic and tubular components. We conclude that gastrointestinal duplications, although rare, should be considered in the differential diagnosis of all abdominal and submucosal cystic lesions and that laparoscopy is a preferred approach for the surgical treatment of gastrointestinal duplications.

Key words: Gastrointestinal duplication; Colonic duplication; Laparoscopy; Intussusception; Congenital abnormalities

Core tip: Gastrointestinal duplications are uncommon congenital malformations and are rarely encountered

in adults. We describe an adult case of ascending colon duplication resected by laparoscopic right colectomy. We conclude that gastrointestinal duplications should be included in the differential diagnosis of all abdominal and submucosal cystic lesions and that laparoscopy is a preferred approach for the surgical treatment of gastrointestinal duplications.

Kyo K, Azuma M, Okamoto K, Nishiyama M, Shimamura T, Maema A, Shirakawa M, Nakamura T, Koda K, Yokoyama H. Laparoscopic resection of adult colon duplication causing intussusception. *World J Gastroenterol* 2016; 22(7): 2398-2402 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i7/2398.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i7.2398

INTRODUCTION

Gastrointestinal duplications are uncommon congenital malformations and are estimated to occur in 1 of 4500 births[1]. These malformations can occur anywhere along the gastrointestinal tract from the mouth to the anus, but the ileum is the most common site (30%-35%) and colonic duplications are rare (7%-20%)[2,3]. Most cases (67%-80%) are recognized before the age of 2 years as an acute abdomen or bowel obstruction, and those condition is rarely encountered in adults[2,3]. We describe here a case of adult colon duplication that caused intussusception and was treated by laparoscopic resection.

CASE REPORT

A 20-year-old otherwise healthy male was referred to our hospital because of a one-year history of intermittent right flank pain and a large submucosal mass in the ascending colon that had been observed by colonoscopy at a clinic. All laboratory tests performed resulted in values within the normal range. An abdominal computed tomography scan revealed intussusception of the right colon with a cystic mass 4 cm in diameter. Although stool-like material was observed within the mass, it did not lead to the diagnosis (Figure 1A and B). A barium enema showed a large submucosal mass in the ascending colon that originated near the ileocecal valve (Figure 2). Colonoscopy also revealed a large submucosal mass in the ascending colon, and two mucosal defects were observed on its surface (Figure 3). Histological examinations of biopsy specimens taken from one of the mucosal defects revealed only necrotic tissue. No other abnormalities were found in either physical or radiologic examinations. A neoplastic submucosal tumor causing intussusception was suspected, and a laparoscopic right colectomy with ileocolic anastomosis was performed. Intraoperatively, a round mass was observed in the hepatic flexure of the colon that caused an invagination of the ileocecal region into the ascending colon (Figure 4). No other abnormalities such as Meckel's diverticulum or malrotation were found. Careful inspection of the resected specimen revealed not only a cystic submucosal mass 4 cm in diameter but also a submucosal tubular bulge measuring 5 cm in length and extending from the mass to the ileocecal valve (Figure 5A). Both the cystic and tubular parts of the lesion were on the mesenteric side. Histopathologically, both the cystic and tubular parts of the lesion were lined by with normal colonic mucosa; the cystic part had its own muscular layer, whereas the tubular part shared the muscular layer with the native ascending colon, which led to the diagnosis of colon duplication (Figure 5B-D). Both of the mucosal defects on the cystic lesion were communication sites with the native bowel, and the cystic lesion contained fecal material and blood clots. No neoplastic lesion or ectopic mucosa was identified. The postoperative course was uneventful, and the patient was discharged on post-operative day 7.

DISCUSSION

In 1876, Suppinger reported the first case of this congenital malformation, and in 1937, Ladd described in detail the clinical and pathological aspects of the lesions and recommended using the term "duplications of the alimentary tract"[4]. These lesions have 3 defined features: (1) well-formed smooth muscle layers; (2) an epithelial lining consisting of some portion of the alimentary tract; and (3) contiguity with a portion of the alimentary tract. There may or may not be communication with the gastrointestinal tract, and the dividing septum may be muscular or merely a double layer of epithelium[5]. Additionally, these malformations may be found in the intermuscular, submucosal or subserosal layer of the intestinal wall and are typically located on the mesenteric side of the bowel[6]. Duplications are divided into cystic (86%) and tubular types (14%), and the cystic lesion usually does not communicate with the lumen of the adjacent bowel and contains a sticky mucoid fluid that is chocolate or *café au lait* colored in some instances and almost colorless in others[2,4,7]. Tubular lesions often have one or more direct communications with the adjacent bowel and can appear either as double-barreled or Y-shaped forms. Gross *et al*[7] described four variations of the duplications: (1) a tubular structure that branches out from the intestine and extends for some distance between the mesenteric leaves; (2) a double-barreled structure communicating with the intestinal lumen at one or both ends; (3) a cystic structure lying free in the peritoneal cavity, attached only by a thin mesenteric stalk (this type is rare); and (4) most common, a spherical lesion contiguous with some part of the bowel, particularly along the ileum. Although both cystic and tubular parts were observed in the present case, we think it plausible that stool accumulation in the tubular duplication caused cystic

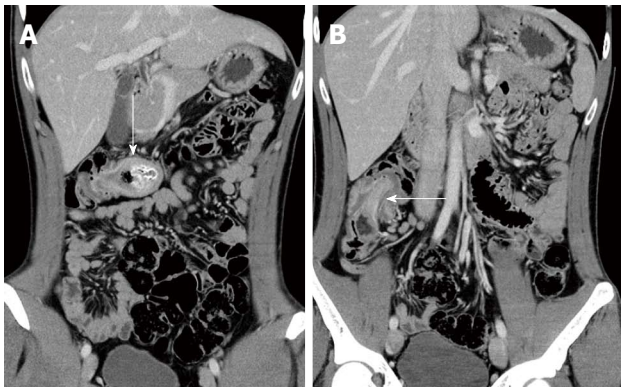

Figure 1 Coronal computed tomography of the abdomen. A: A cystic lesion 4 cm in diameter in the transverse colon (arrow). The lesion contained stool-like material and acted as a lead point for intussusception; B: Intussusception of the right colon (arrow).

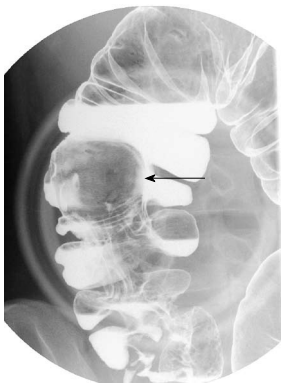

Figure 2 Barium enema. A large submucosal mass (arrow) in the ascending colon originating near the ileocecal valve.

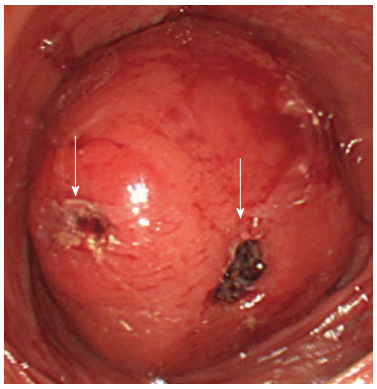

Figure 3 Colonoscopy. A large submucosal mass in the ascending colon. Arrows indicate mucosal defects on the surface of the mass.

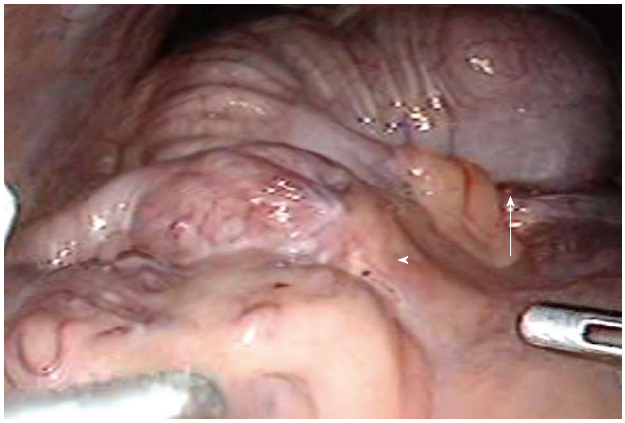

Figure 4 Intraoperative laparoscopic view. A round mass in the hepatic flexure of the colon (arrow) causing an invagination of the ileocecal region into the ascending colon (arrowhead).

dilatation that protruded into the intestinal lumen over the years; therefore, this case should be classified as a double-barreled tubular structure type. Similar cases have been reported in which the end of a Y-shaped tubular colonic duplication had become a large cystic mass containing stool[8,9].

Signs and symptoms of the duplications are nonspecific and depend on their location; oral and esophageal lesions may cause dysphagia and respiratory difficulties, and other intra-abdominal types may cause an abdominal mass, pain, constipation, intestinal obstruction, volvulus, intussusception, hemorrhage or perforation[2,6,7,10]. Hemorrhage often occurs from the ectopic ulcerating gastric mucosa in the duplication. Ectopic gastric mucosa was observed frequently in the duplications irrespective of their locations in the gastrointestinal tract (20%-50%) and was particularly common in esophageal duplications[2,3,6]. The occurrence of carcinoma in a duplicated cyst has also been reported, and 67% of malignancies occurred in colonic duplication, although colonic duplications are relatively rare[11].

Although no other abnormalities were found in the present case, 80% of patients with tubular colonic duplication have been reported to have associated anomalies; urogenital duplications are the most notable, and skeletal abnormalities, including malformations or duplications of the spine and sacrum, are also often observed[10,12]. The etiology of intestinal duplications and the associated anomalies is poorly understood, although several theories have been proposed[10].

The treatment of choice for duplications is surgical excision. Although the treatment strategy for asymptomatic cases is not clearly established, surgical excision is generally recommended to prevent the complications described above and the occurrence of malignancies. However, complete excision may not be possible for a long tubular duplication. In such a case, selective mucosal excision with preservation of the seromuscular layer[6,10,13] or distal internal drainage by excision of the common wall may be effective alternatives[2,3,10]. Although some recent studies have recommended the use of laparoscopy for the diagnosis and surgical treatment of gastrointestinal duplications, few have reported its use in surgery for duplications[2,14]. We believe that laparoscopic surgery, if possible, is a preferred approach because of its

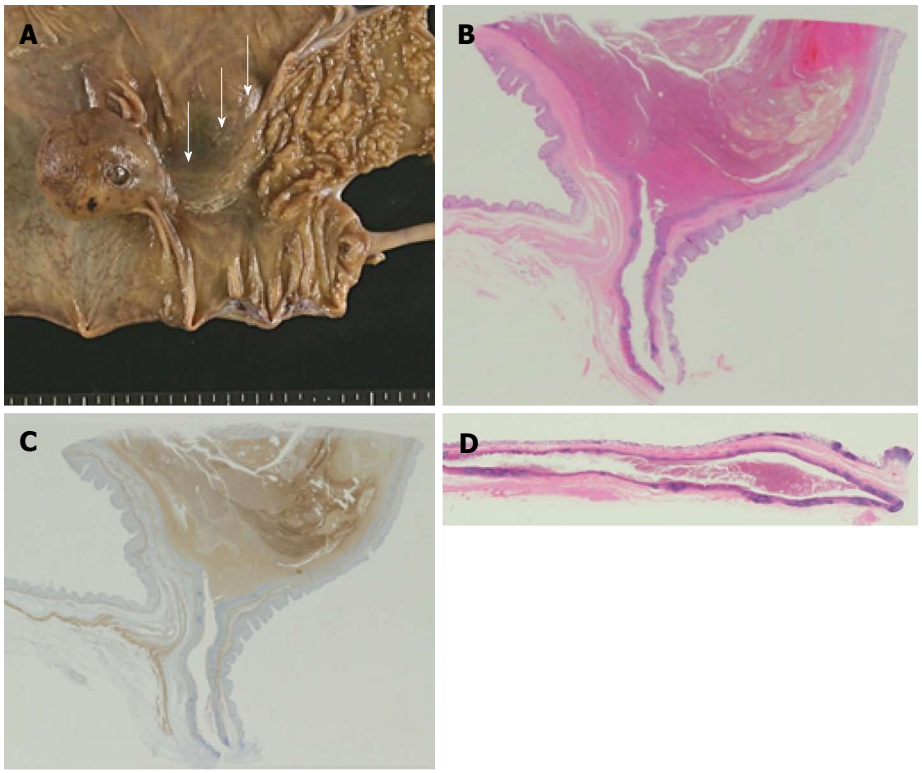

Figure 5 Pathological analysis. A: A macroscopic view of the resected specimen. A round mass 4 cm in diameter is observed in the ascending colon. A submucosal tubular bulge measuring 5 cm in length extends from the round mass to the ileocecal valve (arrows); B and C: Magnified sectional views of the cystic lesion (loupe images; B: hematoxylin and eosin; C: desmin). The cystic lesion is lined by normal colonic mucosa and possesses its own muscular layer; D: A magnified sectional view of the tubular bulge (loupe image; hematoxylin and eosin). A blind-end tubular structure extends from the cystic lesion toward the proximal side to the ileocecal valve. This structure is lined by normal colonic mucosa and shares the muscular layer with the native ascending colon.

minimal invasiveness.

In conclusion, although gastrointestinal duplications, particularly adult cases are rare, they should be considered in the differential diagnosis of all abdominal and submucosal cystic lesions, and laparoscopy should be a preferred approach for the surgical treatment of gastrointestinal duplications. The differential diagnosis of other gastrointestinal cystic lesions can include heterotopic pancreas, lymphangiomas, cystic degeneration of solid tumors such as gastrointestinal stromal tumors and schwannomas, Brunner's gland hyperplasia and gastritis cystica profunda[15-17]. The majority of duplications reported in the literature were unsuspected before surgery, and the diagnoses were established during or after the surgery. It is difficult to make a correct diagnosis without knowledge of the disease, and practitioners should be familiar with the characteristics of this disease.

COMMENTS

Case characteristics

A 20-year-old male was referred to our hospital because of a one-year history of intermittent right flank pain and a large submucosal mass in the ascending colon which had been observed by colonoscopy at a clinic.

Clinical diagnosis

Intermittent right flank pain.

Differential diagnosis

A submucosal tumor of the ascending colon.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

CT showed intussusception of the right colon with a cystic mass 4 cm in diameter.

Pathological diagnosis

Ascending colon duplication.

Treatment

Laparoscopic right colectomy.

Related reports

Gastrointestinal duplications are uncommon congenital malformations, and those encountered in adults are rare. There are only a few reports on laparoscopic surgery for this condition.

Term explanation

Gastrointestinal duplications are uncommon congenital malformations that possess 3 defined features: (1) well-formed smooth muscle layers; (2) an epithelial lining consisting of some portion of the alimentary tract; and (3) contiguity with a portion of the alimentary tract.

Experiences and lessons

Although gastrointestinal duplications, particularly adult cases are rare, they should be included in the differential diagnosis of all abdominal and submucosal cystic lesions.

Peer-review

This article presents a rare case of adult colon duplication that was treated by laparoscopic resection.

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