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EDITORIAL

Colorectal cancer in young adults: A difficult challenge

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Abstract

Sporadic colorectal cancer (CRC) is traditionally diagnosed after the sixth decade of life, and current recommendations for surveillance include only patients older than 50 years of age. However, an increasing incidence of CRC in patients less than 40 years of age has been reported. This occurrence has been attributed to different molecular features and low suspicion of CRC in young symptomatic individuals. When confronting young-onset CRC with older patients, issues such as biological aggressiveness, stage at diagnosis and clinical outcomes seem to differ in many aspects. In the future, the identification of the molecular profile underlying the early development of sporadic CRC will help to plan tailored screening recommendations and improve management. Besides that, differential diagnosis with CRC linked with hereditary syndromes is necessary to provide adequate patient treatment and family screening. Until we find the answers to some of these doubts, doctors should raise suspicion when evaluating an young adult and be aware of this risk and consequences of a late diagnosis.

Key words: Colorectal cancer; Young age; Hereditary; Prognosis

Core tip: Colorectal cancer (CRC) is traditionally considered a disease affecting people with more than 50 years of age. However, numerous researches have detected a rising incidence of CRC in young people, mainly rectal cancer. This finding raises the need for increasing clinical suspicion when evaluating symptoms of a young patient. Furthermore, these groups of patients must be aware of this possibility.

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INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal neoplasia. As it is widely considered a

disease that affects people after the 5th decade of life, screening is not indicated before 50 years of age, when the risk is lower[1]. Over the past years, the increasing use of screening colonoscopy in adults aged 50-75 years has declined CRC incidence by at least 4% per year due to resection of adenomas, also improving the detection of more early lesions[2].

The finding of CRC in adolescents or young adults has always raised attention due to issues such as the emotional impact at diagnosis, the disease behavior and the possibility to be associated with genetic diseases[3].

However, the definition of age to be considered young is controversial. In an analysis of 6425 patients from 55 publications, O'Connell *et al*[4] found that 37 manuscripts considered young those below 40 years of age, while 14 (25%) and 4 (7%) defined as young those below 30 and 35 years of age, respectively. In the literature, most publications refer the CRC incidence in patients with less than 40 years of age[4-6].

The incidence of CRC diagnosed before 40 years of age varies from 0.8% to 15%[5]. Series published during the last decade reveal great variation due to biases associated with experiences from one institution or reference centers. Recently, a greater incidence of young age CRC has been documented[7]. Within this group, a proper investigation to discharge hereditary forms of CRC is fundamental.

So far, age is not universally accepted as an independent prognostic factor. Otherwise, CRC diagnosis in young people is always difficult, as both patients and physicians underestimate symptoms and postpone diagnosis and management[6].

EPIDEMIOLOGY

CRC lifetime risk is around 5%[8]. Since 1998, the CRC overall incidence has decreased in the United States, as a probable effect of CRC screening[9]. However, in a contrast to these overall trends, this pattern is different among young adults.

Studies from *Surveillance Epidemiology and End Result* have presented important findings regarding the epidemiologic trends of CRC in different age groups. Bailey *et al*[10] reported a growing incidence rate (10%) among patients from 20 to 50 years and a 20% reduction after 50 years. Using resource data from 1987 to 2006, Davis *et al*[11] found raising incidences within patients aged 20-49 years, especially among those between 40-44 years (from 10.7 in 1988 to 17.9 per 100 thousand in 2006). From 1973 to 2005, Meyer *et al*[12] identified 7661 CRC patients with less than 40 years in a retrospective study. Age variation throughout time revealed stable rates for colon and increased rates for rectal cancer. Consequently, the authors emphasize that symptomatic young adults should be endoscopically investigated to avoid a late diagnosis, as malignant tumors in young predominates in segments distal to the splenic flexure.

Also, data from the American Cancer Society also noticed an increase in the global incidence from 1992 to 2005 among adults between 20 and 49 years, demonstrating a 3.5% increase per years among men and 2.9% per year in women[13]. More recently, a report from the National Cancer Database identified a consistent incidence increase from 1998 to 2007 by analyzing a group of young-onset (64068, 11%) and later-onset (52480, 89%) CRC[14].

CLINICO-PATHOLOGICAL AND GENETIC ASPECTS

There exists lots of discussion regarding the disparities related to age at CRC diagnosis and tumor biology, recurrence rates, treatment and outcomes. Most publications emphasize that the incidence of young-onset CRC has increased mainly between 40-49 years, when they are more likely to be found in the distal colon and rectum and also advanced stages at presentation.

Although controversial, young patients have been considered to have a more aggressive biological behavior and worst prognosis[15-17]. Furthermore, It has been also described a greater prevalence of mucinous and less differentiated tumors[18] within this group, characteristics also associated with bad prognosis. For these reasons, the question of considering 40 years as the basis for colonoscopic surveillance has been a constant matter of debate, mainly for men[19].

This behavior is generally attributed to the discovery of a more advanced disease, as stages Ⅲ and Ⅳ predominate among the young[14,18,20]. Our group evaluated the question if a late diagnosis could result from less diagnostic efforts in an apparent health group, and we found that symptoms duration was equal (13.8 mo *vs* 14.5 mo, $P = 0.5$) among the young and control group[5]. Others[21] have similarly emphasized that the greater proportion of patients with advanced-stage could not be simply explained by delay in diagnosis.

These results suggest that identification of high-risk young people for screening and recognition of alert symptoms is now a real medical problem. For this, complaints such as persistent rectal bleeding, abdominal pain or anemia should require endoscopic work-ups even in average-risk young people.

One of the main challenges in a young population is to distinguish sporadic from the hereditary forms of CRC. Overall, only 2%-5% of CRC are caused by highly penetrant genes[22], and 15%-20% of CRC in young age population are hereditary[23,24]. An additional problem is that a familiar history of CRC in almost one fifth of hereditary syndromes patients is not recognized[14,25]. Besides this, when diagnosed with a CRC, a young patient should undergo genetic tests, even in the absence of clinical phenotype or normal MSI-IHC studies[26].

So far, knowledge regarding the molecular features of sporadic young-onset CRC is limited. It is generally thought that they derive from a cumulative effect of multiple genetic variants displaying variable penetrance. Probably, the molecular profile in young is heterogeneous and different from late-onset CRC patients[27].

FINAL COMMENTS AND PERSPECTIVES

Important information may be extracted from the present data. Within a young group, CRC is usually diagnosed later and potentially associated with worst prognosis. Thus, a greater suspicion rate is necessary when evaluating young patients with common symptoms. Moreover, educational and preventive programs should provide adequate information regarding alert symptoms and risk populations.

The recognition that CRC incidence at an early age may be correlated with modern dietary factors and epidemic obesity requires improvement in medical awareness and population information about these risk factors[28]. Moreover, young people may develop sporadic cancers that are not usually detected through current screening programs, suggesting a possible drawback in the CRC screening recommendations adopted so far. This fact raised the idea to lower the screening age of those not associated with a high risk (although there is no consensus about it so far), even though the impact of such attitude on early detection, costs and survival has not been fully appreciated yet[29]. In addition, there are lots of barriers to overcome in terms of adherence to preventive colonoscopy at a young age.

In terms of colonoscopic surveillance for risk groups, both patients and physicians should be aware about the importance of establishing a family pedigree. This is based on previous knowledge and investigations demonstrating that the existence of a young family member or a first-degree relative with CRC is risk factors for advanced lesions (including cancer)[30].

Eventually, young age may affect therapeutic decisions, as patients with sporadic cancers before 50 years have been considered for a subtotal colectomy, although this decision not always translates into a greater survival[31]. Otherwise, the recognition of a hereditary syndrome would certainly support the indication of a total colectomy and suggest familial surveillance, besides the absence of prospective randomized trials comparing extended and segmental resections[32]. This tendency correlates with the fact that the presence of mismatch repair deficiency raises the risk of subsequent metachronous neoplasia[33].

Aside from those involved in Lynch Syndrome, many other genes (APC, KRAS, P53, BRAF) may influence tumor genesis and biology. In this context, mutations in FBXW7 and POLE genes were found to prevail in younger patients with CRC[34]. Thus, the most crucial challenge for the future is the understanding of how genetic profile may affect CRC incidence and outcomes of treatment interventions in different age cohorts.

In the absence of genetic information, however, individual decisions should be taken on the basis of health status, family history, opportunity to undergo postoperative follow-up, quality of anal sphincters and patient consent information.

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EDITORIAL

Precision medicine: In need of guidance and surveillance

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Abstract

Precision medicine, currently a hotspot in mainstream medicine, has been strongly promoted in recent years. With rapid technological development, such as next-generation sequencing, and fierce competition in molecular targeted drug exploitation, precision medicine represents an advance in science and technology; it also fulfills needs in public health care. The clinical translation and application of precision medicine - especially in the prevention and treatment of tumors - is far from satisfactory; however, the aims of precision medicine deserve approval. Thus, this medical approach is currently in its infancy; it has promising prospects, but it needs to overcome numbers of problems and deficiencies. It is expected that in addition to conventional symptoms and signs, precision medicine will define disease in terms of the underlying molecular characteristics and other environmental susceptibility factors. Those expectations should be realized by constructing a novel data network, integrating clinical data from individual patients and personal genomic background with existing research on the molecular makeup of diseases. In addition, multi-omics analysis and multi-discipline collaboration will become crucial elements in precision medicine. Precision medicine deserves strong support, and its development demands directed momentum. We propose three kinds of impetus (research, application and collaboration impetus) for such directed momentum toward promoting precision medicine and accelerating its clinical translation and application.

Key words: Precision medicine; Clinical translation; Development; Targeted therapy; Immunotherapy

Core tip: Precision medicine aims toward accurate, efficient and effective diagnostic testing and precise treatment. Emerging techniques and therapeutic drugs based on molecular profiling and genomic characteristics will help achieve that goal. Next-generation sequencing is the most frequently used methodology for precision medicine applications; however, proteomics and metabolomics tests are growing in accuracy and ease of use. In terms of applications and outcomes, the benefits conferred by precision medicine are currently insufficient. Present development of precision medicine lacks order. Therefore, precision medicine needs strong support to develop, and a directed momentum is required. We suggest three kinds of impetus (research, application and collaboration impetus) for such directed momentum toward promoting precision medicine and accelerating its clinical translation and application.

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INTRODUCTION

Advancing technology in genomic sequencing has achieved a level of development, whereby a personal genome can now be obtained for under US$1000 and within a day. That is approximately 3 million times cheaper and 3000 times quicker than during the period of the Human Genome Project, which ended in 2003. Toward a deeper understanding of the human genome, among both healthy and sick populations, novel disease taxonomies and new treatment options (such as basket and umbrella trials in oncological therapy) are regarded as promising areas in current medicine. Accordingly, the term "precision medicine" began appearing in the literature. A bill proposed by the United States President Barack Obama in early 2015 was expected to define disease by the underlying molecular characteristics and other environmental susceptibility factors in addition to conventional symptoms and signs.

The overarching theme of precision medicine is to classify disease more precisely, diagnose more accurately, approach treatment more individually, and undertake more specific preventions. Hitherto, almost all these aims have been based on establishing a vast data network that integrates clinical data from individual patients and personal genomic background with existing research on the molecular makeup of diseases. Based on these big data, individual treatments and customized prevention strategies have been generated to guide clinical application, including sickness prevention, early disease diagnosis, precise therapy, and pharmacological development.

Currently, precision medicine is highly valued. Financial support has been provided through policy changes at the national level and it has received enterprise sponsorship, especially in China and the United States. However, the support has been uncoordinated. The development of the emerging discipline of precision medicine needs stronger support and proper guidance and monitoring.

PARADOXICAL STATUS QUO

Disputes and controversies have plagued precision medicine since it was first proposed. Recently, Tannock and Prasad noted the imperfect, unpromising current status of precision medicine[1,2]. In those publications in top medical journals, the authors found that in oncology, treatment choice based on tumor molecular or personal genomic profiling confers scarce clinical benefits to patients in terms of prognosis, survival time, or quality of life. With the approach to precision medicine in recent years, several negative or inferior outcomes have appeared in numerous areas; for example, targeted therapy in oncology and imperfect application of genome sequencing in disease diagnosis. With SHIVA[3], a randomized controlled phase 2 trial of multiple solid tumors compared the efficacy of targeted agents selected based on tumor molecular profiling (using an algorithmic approach) with that of a physician's choice. The SHIVA results showed that median progression-free survival (PFS), the primary endpoint, was almost equally poor in both cases: 2.3 and 2.0 mo, respectively. This outcome illustrates the uncertain prospects for genomic-targeted therapy in the clinical treatment of cancer.

However, the application and development of precision medicine, especially in oncology, should not be disregarded only because of certain negative results. SHIVA was based on a specific treatment-allocation algorithm, and it was unable to assess any drug's efficacy. Several aspects of that algorithm may have interpreted the PFS results as negative. Those aspects include the definition and prioritization of the specified driver molecular alterations (established in a histology-agnostic way according to the data available when SHIVA was designed in 2011). The influence of resistance aberrations and use of targeted therapies were the only options available, which rather consistently affected the identified molecular alteration directly.

There have been unpromising outcomes with some basket trials, in which molecular targeted agents were applied across diverse histologically defined tumors. However, in trials with the same single genomic alteration or some umbrella trials (in which multiple genomic alterations within the same tumor histology

were targeted), the outcomes primarily resulted from inaccuracies with current precision medicine. This in turn originated in the heterogeneity of tumors and constant evolution in the biological process of cancer[4]. Thus, the belief that the specimen "determines" the result appears frequently in genomic research on oncology, *i.e.*, the specimen selected for a sequence determines the genome sequences of the entire tumor mass. Most current molecular targeted agents lack proper target specificity and, thus, are too short in their pesticide effect. Therefore, large datasets and molecular targeted agents with more specificity and efficacy offer much room for development.

CLINICAL DEVELOPMENT OF PRECISION MEDICINE

Prevention

Hitherto, disease prevention has emphasized etiologic treatment and early screening, which are imperfect in directionality and induce waste in manpower, material resources and funds. With the application of more accurate detection methods in the era of precision medicine, classification based on individuals with different genetic, environmental and lifestyle characteristics can be achieved. In the case of oncology prevention, subgroups with high risk factors should have a different prevention strategy from others, *e.g.,* greater frequency of health examinations or even prophylactic treatment. A large-sample study found that tumor suppressor genes in BRCA mutations may produce hereditary breast and ovarian cancer syndrome[5]. Thus, a more rigorous prevention strategy is required for individuals carrying the BRCA mutation since they belong to the high-risk population.

Genome-wide association studies have allowed the nomination of candidate genes related to a given disease, which is helpful in identifying the disease-susceptible population[6]. In patients with inflammatory bowel disease (IBD), over 200 genetic susceptibility loci have been identified and the gut microbiome characterized; that has promoted precise diagnosis, monitoring, and treatment of IBD[7]. A potential contribution of precision medicine is more comprehensive, accurate understanding of morbidity and mortality trends among populations and groups with diverse cancers. Such a development is particularly pertinent to tumor monitoring and targeted prevention.

Diagnosis

Tumor molecular diagnosis has made tremendous advances following improvements in diagnostic techniques. For many years, finite genomic data have been applied to guide diagnosis, inform prognosis, and support treatment decisions with several types of cancers. For example, p53 mutations have been found to be commonly associated with microvascular invasions, which may result in micrometastasis, followed by frequent recurrences and poor prognosis in hepatocellular carcinoma[8].

Tumor tissue genetic sequencing has gradually become a common test in many medical centers. Circulating tumor DNA detected in the peripheral circulating blood (termed a liquid biopsy) has been validated as significant with various types of cancer; it has been especially promising in early detection, targeted agent selection, drug resistance analysis, and tumor recurrence monitoring in colorectal and lung cancer[9,10]. Other forms of liquid biopsy (including circulating tumor cells, the exosome, and circulating nucleic acid detection) provide minimally invasive methods to diagnose and evaluate cancer status.

This progress in precise diagnosis - alone or combination with conventional examinations - has improved specificity and sensitivity in cancer diagnosis, allowing early detection. Based on divergence in multi-omics combined with conventional cancer definitions, precision medicine has the potential to achieve a new type of diagnostic categorization using unique tumor genetic profiling molecular variations, and biomarker characteristics.

Treatment

The genetic background and expression models of tumors have become clearer; it has become possible to differentiate the mutated genes in tumors from those in adjacent benign tissues. Large-scale sequencing studies have recognized genomic alterations, which are currently used to guide targeted therapy. However, another problem concerns the difficulty in identifying driver genes with gatekeeper mutation in individual genetic profiling. Driver genes have been located; however, only a small proportion of mutation genes possess specific molecular targets for drugs. Owing to the lack of proper understanding of the mechanisms of action of targeted molecular agents, targeted therapy has shown poor efficacy in many tumor patients.

Accordingly, precise classification for all cancer patients has not been achieved, and there is much room for improvement. Even so, precision medicine has heralded the dawn of oncological therapy. For example, hepatocellular carcinoma is worldwide one of the most lethal cancers, with high morbidity in South Asia. The only targeted drug for this carcinoma is sorafenib, which was approved by the United States Food and Drug Administration. Following dedicated genomic profiling in recent years, several targeted drugs, such as regorafenib[11] and lenvatinib[12], have been identified and verified. Those drugs will be used instead of sorafenib and improve the therapeutic efficacy in patients with advanced hepatocellular carcinoma.

Treatment will become more precise and effective through proper, beneficial screening of patients, rather than adopting a unified therapeutic strategy for

patients with the same cancer. This may be achieved by genome sequencing to identify therapeutic targets and more reliable molecular classification of a tumor, as well as broadening the utility of existing molecular targeted drugs in precision oncology.

One of the main goals of precision medicine is maximizing the therapeutic effect and minimizing the occurrence of adverse drug reactions by identifying the correct agent and dose for each patient. Numerous advances in precision medicine have been made in determining and understanding how such factors as genetic polymorphisms influence drug pharmacokinetics (PKs) and contribute to variable drug responses (VDRs). For example, alcoholic liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD), the most common chronic liver diseases in Western society, have been demonstrated to possess a close relationship with intestinal microbiota compositions. Studies have described how products secreted by microbiota-derived metabolites could influence the PKs and VDRs of drugs used for treating ALD and NAFLD[13]. With deeper understanding of host-microbial interactions, novel therapeutic methods targeted at gut microbiota could enable new treatment options to restore the intestinal ecosystem and cure liver disease.

THREE KINDS OF IMPETUS TOWARD DIRECTED MOMENTUM

Precision medicine is currently weak; that has restricted its clinical application and popularity. Negative outcomes in personalized therapy based on individual molecular profiling have also been discouraging for precision medicine. However, the objective pursued by precision medicine deserves approval, and a collaborative impetus is necessary to promote it. Precision medicine pursues its aim based on genetic, biomarker, phenotypic and psychosocial characteristics toward a therapy that targets the needs of individual patients; it distinguishes one patient from other patients with similar clinical presentations. Achieving precision medicine requires a directed momentum. Impetus from diverse aspects should be emphasized as follows.

Research impetus

The orthocenter of precision medicine should not be situated merely in oncological research; it should expand to other disease categories, such as contagious and rare diseases, chronic illnesses, and even among healthy populations. Studies in precision medicine must establish a foundation to construct a reliable biological information database, including proteomics, metabolomics, genomics, epigenomics, and even mobile health technology, that underpins all humanity. Such a development needs a broad research plan to encourage creative approaches to precision medicine.

However, multiple issues hamper this process; they include reluctance to collaborate among professional societies, clinicians and reimbursement organizations. There is a lack of robust criteria to accommodate strict requests for prospective controlled clinical trials, aiming to verify the superiority of an individualized approach for drug prescription based on personal molecular profiling. There is likewise a lack of a reliable reference method for comparing a patient's genome with the greater population's "normal" genome.

Thus, research has emphasized empowering clinicians, patients and investigators to work together toward more personalized care, improving clinical outcomes, and establishing an effective patient cohort, wherein both clinical and multi-omics[14] data are collected.

Application impetus

The clinical application of precision medicine is not limited to molecular targeted therapy and personal genomic sequencing. Precision medicine possesses broader connotations, which include omics analysis, molecular detection, biomarker promotion, molecular imaging, and molecular pathology. Those areas depend on further advances in medical informatics and bioinformatics. Precision medicine will work effectively when the medical establishment integrates research to provide evidence for constant, iterative improvement of medical practice in different disciplines.

Personalized treatments and methodology in precision medicine can also complement conventional therapy and novel immunotherapy, such as immune checkpoint inhibitors in cancer treatment enhancing the clinical benefits. Using whole-exome sequencing, Le *et al*[15] revealed that colorectal cancers with amounts of somatic mutations induced by mismatch repair defects are more susceptible to immune checkpoint blockade with pembrolizumab (a PD-1 antibody). Van Allen *et al*[16] analyzed whole exomes from 110 patients with metastatic melanoma; they found that overall mutational load, neoantigen load, and expression of cytolytic markers in the immune microenvironment were highly correlated to the response of CTLA-4 inhibitor.

Following genomic detection in a population showing inconsistent responses to the same chemotherapeutic agents, molecular profiling has been confirmed to be related to chemotherapy sensitivity. Although this correlation is not strong enough to inform drug selection in all diseases, personalized therapy has been successfully achieved in some patients. Applying the methodology of precision medicine based on traditional therapy and immunotherapy may therefore help uncover discrepancies in populations and locate the sources of different curative effects. These discoveries could translate to enhancers or reinforcing agents overcoming inefficiency and resistance with existing therapies.

Collaboration impetus

The development of precision oncology does not involve repudiation of evidence-based medicine. Almost all present clinical applications of precision medicine refer to evidence-based medicine. To explore the real effects of applying precision medicine to the individual, precise medical practice and validation need to be combined with methodology analogous to real-world research[17].

In the era of precision medicine, robust and thorough supervision should be implemented - especially in commercial genomic sequencing and individual genome consulting. To make the market more canonical and accelerate the merging of precision medicine with evidence-based medicine, related administrations and organizations need to devise detection standards and industry norms. Governments should be responsible for formulating policies that ensure the sound development of precision medicine, leading to virtuous competition, accelerated clinical translation, and providing sufficient freedom to promote cooperation among courses. To maximize the benefits of precision medicine, pharmaceutical companies should undertake more clinical trials and drug development for treating infrequent cancers, such as cholangiocellular and vascular endothelial carcinoma.

CONCLUSION

There is a significant gap between precision medicine and its application in clinical settings and for disease prevention. However, precision medicine is not illusory for medical development; its essence is exploring novel technology to assist with acute interventions in individual patients by generating and using numerous sources of personal data to group and treat patients more accurately. Pursuing precision medicine demands directed momentum based on the impetus from research, application, and collaboration. All players and aspects - academic, social, policy, and economic - should coordinate toward promoting precision medicine.

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REVIEW

Barrett's oesophagus: Current controversies

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Abstract

Oesophageal adenocarcinoma is rapidly increasing in Western countries. This tumour frequently presents late in its course with metastatic disease and has a very poor prognosis. Barrett's oesophagus is an acquired condition whereby the native squamous mucosa of the lower oesophagus is replaced by columnar epithelium following prolonged gastro-oesophageal reflux and is the recognised precursor lesion for oesophageal adenocarcinoma. There are multiple national and society guidelines regarding screening, surveillance and management of Barrett's oesophagus, however all are limited regarding a clear evidence base for a well-demonstrated benefit and cost-effectiveness of surveillance, and robust risk stratification for patients to best use resources. Currently the accepted risk factors upon which surveillance intervals and interventions are based are Barrett's segment length and histological interpretation of the systematic biopsies. Further patient risk factors including other demographic features, smoking, gender, obesity, ethnicity, patient age, biomarkers and endoscopic adjuncts remain under consideration and are discussed in full. Recent evidence has been published to support earlier endoscopic intervention by means of ablation of the metaplastic Barrett's segment when the earliest signs of dysplasia are detected. Further work should concentrate on establishing better risk stratification and primary and secondary preventative strategies to reduce the risk of adenocarcinoma of the oesophagus.

Key words: Barrett's oesophagus; Gastroenterology; Endoscopy; Oesophageal adenocarcinoma; Dysplasia

Core tip: Oesophageal adenocarcinoma is increasing in incidence especially in Western populations. Barrett's oesophagus is the identifiable pre-malignant condition which allows periodic surveillance and secondary prevention to be undertaken to reduce cancer risk. There has been recent evidence supporting earlier endoscopic intervention for dysplastic changes in Barrett's oesophagus, but the high burden of surveillance prompts increased efforts to identify individuals at highest cancer risk to concentrate resources on those patients who will derive the greatest benefit.

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INTRODUCTION

Barrett's oesophagus is an acquired oesophageal condition characterized by the presence of metaplastic columnar epithelium in the distal oesophagus which replaces normal stratified squamous mucosa[1].

It is associated with prolonged gastro-oesophageal reflux and a risk of development of adenocarcinoma of the oesophagus[1]. Diagnosis is made by oesophagogastroduodenoscopy and biopsy sampling to allow histological examination of the oesophageal mucosa (Figure 1). There is a histological spectrum of appearances of the Barrett's epithelium spanning benign changes to adenocarcinoma which is classified using the Modified Vienna Criteria into one of five categories[2] (Table 1).

Following initial diagnosis and confirmation of the histological findings[3-6], the management of Barrett's oesophagus will include consideration of periodic surveillance of the Barrett's mucosa, measures to control gastro-oesophageal reflux, chemo-protective strategies, ablation of the metaplastic segment, endoscopic resection and surgical resection of the oesophagus.

AREAS OF DEBATE

Six decades have passed since Normal Barrett described this eponymous condition[7], yet there are still several areas of controversy surrounding definitions, formal diagnostic criteria, the role of screening, the scope of primary and/or secondary prevention and how to undertake surveillance. The pathogenesis of Barrett's oesophagus is also debateable but will not be addressed fully in this article as the focus is clinical. The most controversial areas surround the fact that overall risk has been established but calculating individualised risk is limited as it is based on crude markers of perceived risk. Also, new evidence for earlier ablation of dysplasia has changed the goals of surveillance; the best risk modification to reduce the risk of dysplasia is not widely practiced; sampling error and pathological interpretation are subject to significant errors; and adjuncts to these methods not being widely taken up.

DEFINITIONS IN BARRETT'S OESOPHAGUS

The definition of Barrett's oesophagus is controversial. There are several definitions of Barrett's oesophagus and without comprehensive population-based studies it is difficult to define the true incidence of the disease. Overall, it is fundamentally the presence of metaplastic columnar epithelium in the distal oesophagus.

However, other factors which are not part of the definition are oftentimes included in the requirements for consideration in surveillance including the precise location of the oesophageal landmarks, the required extent of metaplastic mucosa and the presence of intestinal metaplasia.

Different studies use different clinical end points when examining outcomes in Barrett's oesophagus. Some use cancer incidence or mortality, whereas others use the development of dysplasia (to improve study power or due to therapeutic interventions). Interventional trials may use endpoints such as macroscopic eradication of columnar mucosa, a reduction in Barrett's extent or absence of intestinal metaplasia or dysplasia on biopsies. There is also considerable variability in biopsy protocol and histological grading of biopsy findings.

SCREENING FOR BARRETT'S OESOPHAGUS

Screening identifies the possible presence of disease in asymptomatic individuals to facilitate earlier intervention and management with the aim of reducing morbidity and mortality. The criteria required for a valid screening programme are listed in Table 2.

WHAT IS THE PREVALENCE OF BARRETT'S OESOPHAGUS?

Several studies have established that the prevalence of Barrett's oesophagus in the unselected general population is between 1%-2% in European studies (Italian 1.3%, n = 1033 and Swedish 1.6%, n = 1000)[8,9]. It is 5.6% in the United States[10]. The factors associated with Barrett's oesophagus are gastro-oesophageal reflux disease (GORD) symptoms[11-16], older age[11-13], and the male gender[11,12,17]. Studies have revealed an association with central obesity (waist to hip ratio or abdominal circumference, but less clearly to body-mass index or overall body fat content), tobacco smoking, the Caucasian race and a positive family history. Conversely, alcohol consumption does not appear to be a strong risk factor. Studies have also found potential risk factors including metabolic syndrome, type 2 diabetes mellitus, and sleep apnoea[18-21]. It has been suggested that the difference in prevalence between the United States and Europe is due to a higher prevalence of associated risk factors (GORD, obesity, diet, smoking); and can explain the reason behind the difference in prevalence between the West and Asia or Africa[8,22]. Nevertheless, data from meta-analyses on the difference in cancer

Table 1 Modified Vienna Criteria

Category	Description
1	No dysplasia
2	Indefinite for dysplasia
3	Low-grade intraepithelial neoplasia (low-grade adenoma/dysplasia)
4	High-grade intraepithelial neoplasia (high-grade adenoma/dysplasia, non-invasive carcinoma, or suspicion of invasive carcinoma)
5	Invasive epithelial neoplasia (intramucosal carcinoma, submucosal carcinoma, or beyond)

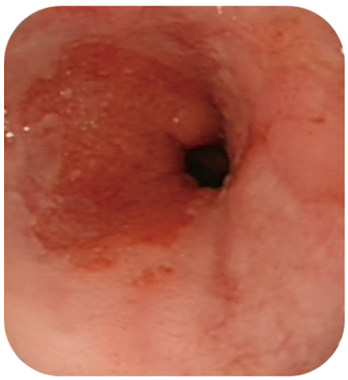

Figure 1 Barrett's oesophagus on endoscopy.

incidence between countries across the world do not show a difference in cancer risk[23]. However, there are likely to be differences between individual studies so further individualised risk stratification is needed with a possible inclusion on geographical location.

In examining the risk of development of oesophageal adenocarcinoma in the general population: a large case-control study found that the OR of developing oesophageal adenocarcinoma for patients with GORD symptoms at least once a week was 7.7 (95%CI: 5.3-11.4) compared to individuals without GORD symptoms[24].

In summary, Barrett's oesophagus is an important health problem as it is an identifiable premalignant leading to oesophageal adenocarcinoma[1]. There is a detectable early stage where an effective intervention would be more beneficial than at a later stage as it would reduce the risk of malignant progression.

NATURAL HISTORY OF BARRETT'S OESOPHAGUS

There is an asymptomatic but detectable early stage which offers a window for treatment. Treatment of cancer/dysplasia is more beneficial the earlier it is given[25]. Subsequently, the natural history is now often interrupted by interventions made when dysplasia is identified[26]. Evidence for the efficacy of various interventions (endoscopic, pharmacological and surgical) on the natural history is currently being studied.

Two specific major United Kingdom trials currently underway are: the Barrett's Oesophagus Surveillance Study (BOSS) which randomises patients to standard surveillance *vs* endoscopy at time of need and the Aspirin and Esomeprazole Cancer Chemoprevention Trial[27,28] which is discussed in the section on Secondary prevention.

Nevertheless, for now, it is agreed that oesophageal adenocarcinoma develops by a multistep process where a normal stratified squamous cell in the distal oesophagus becomes metaplastic columnar epithelium under the environmental assault of gastric acid, made more likely on a background of genetic and non-modifiable risk factor predisposition[29] and onward to neoplasia (Table 1). The process is dependent on defective genes amongst those which control the cell cycle where genomic instability results in multiple aneuploid populations of cells; which will genetically acquire the ability to invade and metastasize[30-32].

A number of studies have reported resolution of dysplastic changes and whilst regression to a less severe dysplastic stage may be plausible, the absence of dysplasia (which by definition is neoplastic with genetic changes) is more likely to be due to sampling error or variability in histopathological interpretation[30,32-34]. Several papers conclude that the natural history of Barrett's oesophagus is not known with an unpredictable progression[29]. Moreover, attempting to understand the natural history becomes more difficult on an individual patient basis as it would require consideration of genetic, environmental and behavioural factors[35]. Despite the uncertainty, a study in Northern Ireland found that the annual risk of oesophageal adenocarcinoma in patients with Barrett's oesophagus was 0.38% per year (when intestinal metaplasia is present) compared to 0.07% in patients without intestinal metaplasia[36]. The lifetime risk was 5.8% in males and 3.0% in females. Overall, there are many questions surrounding the pathogenesis which require further research into.

TARGETTED SCREENING

A decision analytical model established that a one-time screening endoscopy for Barrett's oesophagus was cost-effective[37]. Nonetheless, there is a debate on the target population for screening and most guidelines advocate targetting individuals with certain risk factors rather than the general population to maximise its yield. One of the important risk factors considered includes GORD. There is evidence lacking for the most suitable tests and potential methods for screening include endoscopy but this is generally considered to be too expensive, invasive and cumbersome where a study found that the cost of endoscopic screening in GORD patients was $24718 per life-year saved[38]. A similar figure ($22200) was also arrived at in another study where the population screened incorporated a number of the key risk factors (50-year-old white men with a history of GORD)[39,40]. Another study

Table 2 The Wilson-Jungner criteria for appraising the validity of a screening programme

The Wilson-Jungner Screening Criteria	Achieved for Barrett's oesophagus?
The condition being screened for should be an important health problem	+
The natural history of the condition should be well understood	+/-
There should be a detectable early stage	+
Treatment at an early stage should be of more benefit than at a later stage	+
A suitable test should be devised for the early stage	+
The test should be acceptable	+
Intervals for repeating the test should be determined	+/-
Adequate health service provision should be made for the extra clinical workload resulting from screening	+
The risks, both physical and psychological, should be less than the benefits	+
The costs should be balanced against the benefits	-

investigating the possibility of streamlining the number of patients requiring endoscopic surveillance has positive results. They limited the surveillance cohort after an initial endoscopy to patients with 2 cm of columnar metaplasia or more, and limited again after the second endoscopy by excluding patients without intestinal metaplasia. Results showed that when the risk was stratified in this way, the percentage requiring endoscopy was reduced by 33% and the procedure becomes cost-effective[41].

Cytosponge

The Cytosponge is a device encased within a pill attached to a piece of string which, when swallowed, dissolves to reveal an expandable sponge which scrapes off up to 500000 cells when withdrawn up the oesophagus by the string[42]. Whilst being withdrawn, it collects cells along the entire oesophagus rather than just point samples from endoscopy. Studies have already shown that the Cytosponge technique is able to overcome the sampling bias of endoscopy and is able to reflect the entire clonal architecture[43].

The initial study (BEST1) of 500 patients between 50 to 70 year olds found that 99% were able to swallow the device without issues. A larger study (BEST2) was conducted involving 1110 patients with Barrett's oesophagus (n = 647) or GORD but not investigations for Barrett's oesophagus (n = 463) where both groups swallowed the Cytosponge (93.9% swallowed successfully) and underwent an endoscopy. Results showed that the Cytosponge was as accurate as endoscopy and was preferred to endoscopy in over 90% of patients[44].

The sensitivity of the device was 79.9% which rose to 87.2% for patients with more than 3 cm of circumferential Barrett's oesophagus. It rose further again to 89.7% when the Cytosponge was swallowed twice during the study (n = 107). Specificity was unchanged (92.4%). The study demonstrated that the Cytosponge is safe and acceptable and comparable to other screening options[40]; however, it was a case-control study rather than a population-based study which limits the amount generalization that can be made to a primary care population[44]. Therefore, the BEST3 Trial is in place to investigate its use in a primary care setting and evaluate its cost effectiveness. A small study found that 16% of 161 endoscopy referrals were suitable for triage to use of the Cytosponge[45].

Targetted population

Cost effective identification of patients at highest risk will involve symptoms, demographics and other associated factors (as previously discussed). A meta-analysis of five case-control studies (1189 oesophageal adenocarcinoma patients and 4666 controls) revealed that patients with weekly GORD symptoms were five times more likely (OR = 4.9) to develop oesophageal adenocarcinoma than their counterparts with less frequent or no symptoms[46]. The question remains as to whether a single (one off) screening test is appropriate or whether repetition should be undertaken. Studies show that the mean age at the time of diagnosis is approximately 55 years[47] and whilst children may have patches of columnar epithelium in the oesophagus or distal oesophageal columnarised segments, it is rare before five years of age[48]. This epidemiology suggests that Barrett's oesophagus is an acquired condition and provides insight into informing the age at which screening should start.

The absolute risk for development of adenocarcinoma in individuals with GORD symptoms less than once per week is very low at 0.1 to 15.4 per 100000 for men (aged 30-80 years), and 0 to 2.3 per 100000 for women (aged 30-80 years)[49]. Over 40% of patients with oesophageal adenocarcinoma do not have a history of heartburn, and a study in 2000 found that fewer than 5% of patients with oesophageal adenocarcinoma were known to have had Barrett's oesophagus before they presented with symptoms[24,50]. Subsequently, a targetted screening (or surveillance) programme will only detect some of the individuals at risk[49].

The lack of utility in screening all GORD patients was echoed in the guidelines from the American Gastroenterological Association (AGA)[6] and the American College of Physicians[45] which state that endoscopy should be offered to patients with risk factors for adenocarcinoma. According to both guidelines, these include chronic GORD, hiatal hernia (Figure 2), age 50 years and over, male gender, Caucasian race,

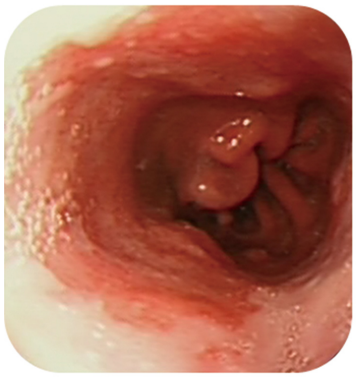

Figure 2 Barrett's oesophagus extending above a hiatus hernia.

and intra-abdominal body fat distribution. Interestingly, the American College of Gastroenterology (ACG)[3] also supports the use of endoscopy as a screening tool but only if there are GORD symptoms in the presence of alarm symptoms (dysphagia, weight loss, and signs of gastrointestinal bleeding). The British Society of Gastroenterology (BSG) also arrived at similar conclusions where it was decided that endoscopic screening is unfeasible and unjustified in an unselected population with GORD symptoms but should be considered in patients with chronic GORD and multiple risk factors (at least three of age 50 years or older, Caucasian race, male sex and obesity)[4]. The report also mentioned that the threshold should be lowered if there is a family history of Barrett's oesophagus or oesophageal adenocarcinoma; and that life expectancy of the individual should be considered in view of screening. The National Institute of Health and Care Excellence also has guidelines on a "two-week wait referral for suspected upper gastrointestinal cancer" where endoscopy is urgently arranged for patients with the following symptoms: dysphagia or aged 55 and over with weight loss and upper abdominal pain, reflux or dyspepsia[51].

DIAGNOSIS AND CONSIDERATION OF ENTRANCE INTO SURVEILLANCE PROGRAMMES

Controversies around the entrance into a surveillance programmes focus on the relevance of intestinal metaplasia and the minimum length of Barrett's oesophagus needed. Following endoscopic and histological diagnosis, as per the BSG, AGA, ACG and American Society for Gastrointestinal Endoscopy (ASGE)[3-6], intestinal metaplasia is not compulsory but helpful, and patients with at least 1 cm of columnar metaplasia of the oesophagus may be considered for surveillance.

Relevance of intestinal metaplasia

There have been differences in opinion on the importance of the detection of in short (arbitrarily defined as < 3 cm) segments when considering the management of Barrett's oesophagus.

Intestinal metaplasia is the transformation of oesophageal or stomach epithelium into that which resembles intestinal epithelium (with goblet cells identified in the sampled columnar epithelium). Its requirement for consideration of surveillance arises from a suspected lower risk of development of oesophageal adenocarcinoma in short segment Barrett's epithelium without features of intestinal metaplasia. Recommendations on surveillance based on the presence of intestinal metaplasia have not been included in previous BSG guidelines[4] due concern that in the process to confirm intestinal metaplasia, there would be limitations from sampling errors in mucosal biopsy samples alongside some studies suggesting it does not influence cancer risk[52]. Moreover, a studies showed that initially the rate of developing dysplasia or cancer was the same in patients with or without intestinal metaplasia[53,54].

Furthermore, in one study which undertook a survival analysis, over 50% of those without intestinal metaplasia initially had evidence of it within 5 years and there was a cancer risk in patients where intestinal metaplasia had not been detected. The study demonstrated that a low number of biopsy samples (fewer than 8) is not enough to exclude intestinal metaplasia, especially if the segment of Barrett's oesophagus is short[55]. It has also been demonstrated that DNA content abnormalities are comparable in both metaplastic epithelia without goblet cells and metaplastic epithelia with goblet cells; however, another study has shown that cancer is commonly found with the surrounding presence of goblet cells[56-58]. Subsequently, a large study from Northern Ireland found that the incidence of high grade dysplasia and cancer in patients with intestinal metaplasia is five times higher than those without intestinal metaplasia (0.38% *vs* 0.07%)[36,56].

The other issue surrounding the use of intestinal metaplasia in the definition is distinguishing between true Barrett's oesophagus and intestinal metaplasia of the cardia of the stomach. It is notoriously difficult to distinguish between the two on a gastro-oesophageal junction biopsy sample as the different forms of intestinal metaplasia occur at both sites and unless native oesophageal structures are seen by the histopathologist, there is a lack of reliable markers which distinguish between intestinal metaplasia of the oesophagus and cardia requiring accurate endoscopic technique when sampling this dynamic organ[59-62].

It thus follows that although intestinal metaplasia is not a prerequisite for the definition of Barrett's oesophagus, it could and should be taken in consideration when determining the frequency and necessity of follow-up of patients as there is evidence that it affects cancer risk long term following the results of the large study from Northern Ireland[36].

1-cm threshold and "long segment" Barrett's oesophagus

Another area of contention is the use of a 1-cm threshold. The use of 1-cm stems from studies which have shown that segments below 1 cm have very high levels of inter-observer variability, and are at very low risk of development of oesophageal adenocarcinoma, and do not show that they are at an increased risk of developing dysplasia[63]. Therefore, they are not considered as Barrett's oesophagus but as "specialized intestinal metaplasia of the oesophagogastric junction", an irregular z-line or "ultra-short segment Barrett's oesophagus". "Long segment Barrett's oesophagus" describes metaplastic segments of 3 or more centimetres in length. Increased segment lengths have been associated with higher dysplasia and cancer risk and subsequently the presence of intestinal metaplasia is not considered a pre-requisite for either diagnosis or enrolment into surveillance programmes[64,65].

PRIMARY PREVENTION

Some of the most important risk factors (male gender, older age) for developing Barrett's oesophagus cannot be modified which limits the scope for primary intervention to preventing GORD by maintaining a healthy weight and not smoking. However, the influence that these risk factors have on the probability of developing Barrett's needs to be investigated to ascertain whether differential efforts need to go into the respective risk factors as smoking confers a greater risk for Barrett's oesophagus in non-GORD controls.

SURVEILLANCE

Oesophageal adenocarcinoma is a tumour which tends to spread early before dysphagic symptoms become apparent with lymph node metastasis being a very poor prognostic factor. The goals of surveillance are to detect dysplasia and early cancer before distant disease has developed[66]. Lymphatic invasion may occur very early in oesophageal tumours (when the tumour has reached the submucosa) which is one of the main reasons for the frequent presentation of advanced disease and poor prognosis[67]. For this reason, there is immense benefit in the early detection of cancer or pre-cancer where intervention may be curative. Tumours detected within Barrett's oesophagus surveillance programmes are in general at an earlier stage than those detected *de novo*[68].

Endoscopy is the main method of surveillance in Barrett's oesophagus with biopsy sampling using the Seattle protocol which consists of four-quadrant biopsies taken every 2 cm or every 1 cm in cases of dysplasia[69]. The purpose of surveillance is to detect dysplasia and at present, the frequency of surveillance is generally based on the grade of dysplasia detected. Prior to surveillance, it is imperative that GORD (if present) is medically controlled as active inflammation makes it very difficult to differentiate between dysplasia and reparation. The biopsies taken should then classed using the five-tier system of the Vienna classification[2] (Table 1).

In most guidelines, surveillance is every 2-5 years if there is no dysplasia, every 6-12 mo for low grade dysplasia (unless an endoscopic intervention has been undertaken) and every 3 mo for high grade dysplasia with most patients undergoing endoscopic therapy rather than continued surveillance. Indefinite changes for dysplasia prompt an early repeat endoscopy (typically at 6 mo) with maximal control of reflux in the interim period to help clarify the histological features and allow more accurate interpretation. The evidence base behind the surveillance intervals arises from decision analytical models which found that surveillance every 5 years was on the only viable strategy with the greatest quality adjusted life[69]; informing the maximal interval of 5 years. Another model found that there was little benefit of surveillance of Barrett's oesophagus patients without dysplasia as there is a low incidence of adenocarcinoma in the group. It demonstrated that if there is no dysplasia, surveillance intervals longer than 5 years are associated with costs outweighing the marginal increases in quality adjusted life years[37].

Observer variability

Surveillance relies on histologic evaluation of dysplasia which unfortunately attracts downsides as there are pathologic limitations and diagnostic variability in assessing the presence and grading of dysplasia[70]. This is worsened by the fact that the changes which occur as Barrett's oesophagus progresses are subtle and accompanied by a wide range of morphological patterns of atypia thus introducing intra- and inter-observer variability[71,72]. The impact of the variability is most obvious at the lower end of the scale where it becomes challenging to differentiate regeneration from dysplasia but not the extremes where agreement is generally very high[73]. A study into the variability found that amongst eight expert oesophageal histopathologists, there was only 60% agreement in drawing a distinction between no dysplasia detected from indefinite changes for dysplasia and low grade dysplasia[74]. The effect is a predilection to a provisional diagnosis of indefinite changes for dysplasia. Various studies have investigated whether the variability in the diagnosis of dysplasia can be decreased but there are yet to be substantial solutions[75]. There is clearly the need for less subjective markers to determine the risk of malignant progression Barrett's oesophagus.

BIOPSY PROTOCOL AND ADJUNCTS TO STANDARD SYSTEMATIC BIOPSY

There have been some studies examining the utility

of a systematic biopsy protocol in comparison to a random or targetted approach[76]. One study found that four-quadrant biopsy detected dysplasia in Barrett's oesophagus in 13 times more patients than non-systematic biopsy surveillance. There is also discussion around the benefit of targetted biopsy samples where adjuncts are used alongside endoscopy to better visualise the oesophageal mucosa.

Chromo-endoscopy

Chromo-endoscopy is founded on the current use of acetic acid to stain abnormal tissues during an examination of the cervix to whiten immature (young) and dysplastic cells. When acetic acid is used in the gastrointestinal tract *via* a spray catheter in the endoscope, both the oesophageal and gastric mucosae turn white (as in the cervix) but once a few minutes have passed, normal mucosa remains white whereas Barrett's mucosa transiently turns red, as does gastric columnar mucosa[77]. Its use can be improved by the addition of indigo carmine to better visualise early gastric cancer and as a mucolytic to remove mucus obscuring the mucosa[78]. Dysplasia may be found where there are areas of surface irregularity, changes in the vascular pattern or variability of staining.

A retrospective study (n = 982) involving patients with Barrett's oesophagus under surveillance found that dysplasia was detected in 41/327 (13%) patients where acetic acid was used as an adjunct *vs* only 13/655 (2%) in the random biopsy group[79]. Moreover, in the initial detection of Barrett's oesophagus, targetted biopsies using acetic acid more than doubles the yield of detection (57% *vs* 26%)[80]. Other studies have shown similar results for acetic acid chromo-endoscopy which was found to detect dysplasia and neoplasia better than white light endoscopy[81], with another study showing that it requires 15 times fewer biopsies per neoplasia detected[79]. In another study where 263 procedures were examined with neoplasia in 143, acetic acid chromo-endoscopy correctly identified 96% of these cases *vs* 55% with white light endoscopy.

Other dyes which have been used include methylene blue, toluidine blue, cresyl violet, crystal violet, Congo red, phenol red and Lugol's solution. Lugol's solution contains potassium iodide and iodine, both of which attach avidly to glycogen in non-keratinised squamous epithelium and so studies have found it is extremely effective for detecting squamous lesions (sensitivity 9% *vs* specificity 40%-95%)[82] and it can also be used in post-ablation Barrett's oesophagus patients to distinguish between regenerative squamous epithelium and areas of residual Barrett's mucosa (which do not take up the dye). Despite its benefits, safety studies have shown that its use may cause retrosternal pain which is attenuated by sodium thiosulfate[83]. Methylene blue is only taken up by tissue which is actively absorbing (small intestinal and colonic epithelium) and so can be used to find Barrett's mucosa (metaplastic absorptive mucosa). Although indigo carmine is predominately used in investigating the colon by visualizing pit patterns to distinguish between different types of polyps, it can also be used to identify Barrett's oesophagus when used in conjunction with high-magnification endoscopy and Lugol's solution[84]. Both cresyl violet and crystal violet stain cell nuclei thus aiding in identifying Barrett's metaplastic mucosa[84,85]. Less commonly used adjuncts to endoscopy are Congo red and phenol red which are both pH indicators used to detect areas of ectopic acid secretion.

Although chromo-endoscopy offers benefits to aid in the screening or surveillance of Barrett's oesophagus, it does have a number of shortcomings that limit its use. Unfortunately, the procedure is very subjective and subject to inter-observer variability and a study found that even when blinding techniques are employed, there was no increase in the numbers of cases of Barrett's oesophagus detected and no widely accepted standardisation of their application[86-90].

Narrow band imaging

Narrow band imaging (NBI) is an alternative technique where lights of specific blue (wavelength = 440-460 nm) and green (wavelength = 540-560 nm) wavelengths are used to enhance the detail of the mucosa and blood vessels. This works because the wavelengths correlate with the peak light absorption of haemoglobin hence will appear very dark thus improving their visibility and easing the identification of neighbouring structures.

The other methods which can be employed for surveillance of Barrett's oesophagus include endosonography [endoscopic ultrasound (EUS)], optical coherence tomography (OCT), confocal microendoscopy, auto-fluorescence endoscopy and computed virtual chromo-endoscopy (CVC).

Endoscopic ultrasound

Studies have shown that EUS to screen patients with Barrett's oesophagus is neither justified nor cost-effective but does play a role when there is high grade dysplasia or intramucosal carcinoma[91]. Conversely, in terms of superiority, OCT is above EUS as its resolution is better as once can see the layers of the oesophageal wall can be visualised with good correlation to histologic structures thus allowing endoscopists to detect high grade dysplasia earlier. The sensitivity of detecting dysplasia was 68% and specificity was 28%[92].

Computed virtual chromo-endoscopy

CVC enhances mucosal surface contrasts and vascular pattern variability without the use of dye as is standard in chromo-endoscopy. Its utility was demonstrated in a randomised control trial where 57 patients with Barrett's oesophagus and a history of high grade intraepithelial neoplasia/early cancer were allocated to undergo acetic acid chromo-endoscopy or CVC with

re-examination after 4-6 wk with the other procedure. The positive predictive value for the former was 39% and 37% for the latter with comparable sensitivities at 83% and 92% respectively[92]. The study thus shows that CVC is not only useful as an adjunct but provides comparable results to conventional chromo-endoscopy in the detection of high grade dysplasia/early cancer.

Auto-fluorescence

Auto-fluorescence endoscopy is a technique incorporating a real time wide angle view allowing the endoscopist to rapidly go from standard white lighting to auto-fluorescence and to very quickly examine large areas of gastrointestinal mucosa. However, there was no clear superiority over conventional white-light imaging (whether or not it is used in conjunction with NBI)[93]. On the other hand, confocal microendoscopy is where the resolution and contrast of imaging is augmented by eliminating out of focus light by the addition of a spatial pinhole at the confocal plane of the lens. A study determined that this method is very accurate and reliable (sensitivity 88% and specificity 96%) for the diagnosis of neoplasia[94].

ENDOSCOPIC SURVEILLANCE INTERVALS

Non-dysplastic (no dysplasia detected)

The BSG, ASGE and AGA are all in agreement on the management which follows for each biopsy category[3-6]. If biopsies show non-dysplastic Barrett's oesophagus (no dysplasia detected), surveillance (every 2-5 years) is offered following a discussion about its benefits and risks. The Barrett's segment length is incorporated into guidelines too. Australian and British guidelines state that endoscopy should be repeated 3-5 years if the maximal length is less than 3 cm, and every 2-3 years if above or equal to 3 cm[4,95]. The AGA and ACG does not delineate surveillance for no dysplasia detected by segment length (3-5 years for all lengths); however, the latter differentiates the number if biopsies by segment length (4 biopsies for every 2 cm of segment length, or at least 8 biopsies if the segment is less than 2 cm at the initial exam) which should reduce sampling error for detection of intestinal metaplasia[53].

Indefinite changes for dysplasia, Low grade dysplasia, High grade dysplasia and Adenocarcinoma

If biopsies are indefinite for dysplasia, American and British guidelines emphasise maximal acid suppression with a PPI to reduce the misleading effects of reflux oesophagitis on the oesophageal mucosa. After adequate acid suppression (BSG and Australian guidelines: 6 mo; ACG: 3-6 mo), further biopsies should be taken using the Seattle biopsy protocol and if they are still indefinite, the diagnosis should be confirmed by an expert oesophageal histopathologist. If the diagnosis is clarified with classification to another group on the second biopsy, the appropriate pathway (no dysplasia detected, low grade dysplasia, high grade dysplasia, adenocarcinoma) should be taken.

If low grade dysplasia or high grade dysplasia/intramucosal carcinoma is seen, the findings must be confirmed with an expert oesophageal histopathologist and the Seattle protocol used to obtain further systematic biopsies (due to the risk of sampling error and confirm the degree of dysplasia) with endoscopic resection of any mucosal irregularities. Guidelines from the United States and the United Kingdom recommend that low grade dysplasia patients are given the option of either surveillance every six months or endoscopic eradication. For high grade dysplasia/intramucosal carcinoma patients, guidelines recommend an intervention to the dysplastic mucosa at this time due to the risk of an occult carcinoma or disease progression[64,96]. Although endoscopic eradication is recommended for high grade dysplasia, endoscopic surveillance is advocated in some units. The evidence comes from a study over a period of 20 years where of 75 patients with high grade dysplasia underwent surveillance over an average of 7.3 years, and 12 developed adenocarcinoma which was curable by ablation in all but 1 who was lost to follow-up[97]. In another study of 45 patients with diagnosed cancer from high grade dysplasia, 13 were detected at the initial endoscopy whereas 32 were found during surveillance and of the 32, only one patient had metastatic disease when first seen on surveillance[98].

Patients who are found to have frank oesophageal adenocarcinoma need to undergo staging investigations with a frank discussion on possible treatment options. The radical options include chemo-radiotherapy, radiotherapy, an oesophagectomy or endoscopic resection/ablation of disease confined to the mucosa. Quality of life is slow to return after an oesophagectomy and not regained in patients surviving less than 2 years[99].

Unfortunately, abiding by comprehensive systematic biopsy protocols is very challenging because there is a substantial time and resource implication to taking multiple biopsies including time to process and interpret results. Moreover, there is no widely utilised system for targetted biopsies.

SURVEILLANCE-DETECTED CANCERS

Overall, studies have already demonstrated that survival rates are markedly better in patients with Barrett's oesophagus where endoscopic surveillance has detected oesophageal adenocarcinoma compared to patients not undergoing surveillance[100-105]. Despite the potential for the findings from these studies to be explained away with lead time and length time biases, the findings were maintained even after correcting for these biases. Nevertheless, there are costs associated with surveillance including the small morbidity associated with surveillance and biopsy, the resource use and associated anxiety. There are

also the limitations associated with surveillance programmes in their goals of detecting dysplasia and early cancer. There is an ongoing randomised control trial (BOSS Trial) comparing survival rates in 3400 patients with Barrett's oesophagus in a standardised 2-year endoscopic surveillance group *vs* an "at need endoscopy" group[27]. Results from the study will contribute towards the settling the debate on the need and benefit of surveillance to cancer incidence or survival.

SECONDARY PREVENTION

Unfortunately, the incidence of Barrett's oesophagus and oesophageal adenocarcinoma are on the rise[46].

Medical control of reflux

Acid suppression with PPIs is a fundamental part of the management of patients with Barrett's oesophagus, and PPIs have been shown to be superior to histamine receptor antagonists[106]. It is known that PPI use relieves symptoms associated with GORD but its effect on the risk of progression to cancer is not known. It has been postulated that if PPI treatment could reduce the stage of dysplasia or the length of Barrett's mucosa, it would contribute to a reduction in the cancer risk[107,108]. At present, studies show that PPI use promotes squamous re-epithelialization next to and on top of Barrett's mucosa but does not cause regression hence surveillance would still be necessary[109-111].

Chemoprevention

There are data that suggest that non-steroidal anti-inflammatory drugs (NSAIDs, particularly aspirin and COX inhibitors) and statins reduce the risk of malignant progression which was seen in a study of 570 Barrett's oesophagus patients who were investigated across 4.5 years[112]. The study demonstrated that the use of both pharmacological agents together had an additive protective effect. These findings and suggestions have been replicated in several other studies supporting the potential implementation of chemoprevention into guidelines[113-118] including one which found that aspirin chemoprevention was more effective and less expensive than endoscopic surveillance alone[119]. However, there are data which suggest the opposite or discuss it in general[120-122]. Table 3 summarises all the studies[113,115-117,121,123]. Nevertheless, at present, the BSG, AGA and ACG guidelines do not recommend chemoprevention.

Anti-reflux surgery

Anti-reflux surgery (fundoplication) has been shown to offer some benefits to patients with Barrett's oesophagus which is mostly symptomatic relief[124-127]. However, at present, there is conflicting evidence with some studies (including meta-analyses) showing that anti-reflux surgery does not reduce the risk or incidence of adenocarcinoma but others do show a lower cancer risk[128-130].

TREATMENT OF DYSPLASIA

In the past, an oesophagectomy was the preferred option for the management of dysplasia in Barrett's oesophagus but nowadays, it can be managed using endoscopic techniques such as ablation or resection. Ablative therapy uses energy to destroy the Barrett's mucosa (without damaging the deeper oesophageal wall) but does not provide a tissue sample.

The most commonly used endoscopic ablative therapy is radiofrequency ablation (RFA) and studies demonstrate that patients with low- and high-grade dysplasia treated with RFA were less likely to undergo malignant progression of their disease than controls[129]. A meta-analysis looking into the efficacy of radiofrequency ablation found that 91% of patients across 20 studies had complete eradication of dysplastic Barrett's mucosa[130]. However, recurrence is an issue as a study of 246 patients with high grade dysplasia or intramucosal carcinoma found that despite initial eradication in 80% of cases, neoplastic recurrence was at 25% by 5 years and metaplastic recurrence was 50% by 4 years[131]. Until recently the role of endoscopic ablation of low grade dysplasia was controversial, but this has changed with the recently published outcomes from the SURF study (Surveillance *vs* Radiofrequency Ablation)[132].

This randomized control trial which compared surveillance with radio-frequency ablation for low grade dysplasia. The trial was undertaken at 9 Barrett treatment centres in Europe where eligible patients had confirmed low grade dysplasia Barrett's oesophagus (seen on endoscopy within the previous 18 mo). Patients were excluded if they had previous endoscopic treatment for Barrett's oesophagus, a history of high grade dysplasia or adenocarcinoma, active secondary malignancy, an estimated life expectancy of less than 2 years, and who were under 18 years or over 85 years. Randomization was in 1:1 ratio into either the ablation group or the endoscopic surveillance (control) group.

The trial found that the ablation resulted in a reduced risk of neoplastic progression (high grade dysplasia or adenocarcinoma) over 3 years of follow-up [high grade dysplasia: 1.5% ablation group (n = 1) *vs* 26.5% control group (n = 18), $P < 0.001$; and adenocarcinoma: 1.5% ablation group (n = 1) *vs* 8.8% control group (n = 6), $P = 0.03$][132]. The number needed to treat to prevent one case of high grade dysplasia was 4.0 and adenocarcinoma was 13.6. Moreover, the dysplasia and intestinal metaplasia were completely eradicated and remained so in the majority of patients in the ablation group. The data effectively suggests that ablative treatment is superior to endoscopic surveillance in patients with Barrett's oesophagus and low grade dysplasia. Nevertheless, no patient in the control group had unresectable cancer or cancer-related death.

Table 3 Studies investigating chemoprevention in Barrett's oesophagus

Ref.	Type	Sample size	Chemo-prevention	Effect on risk	Overall
Nguyen *et al*[113], 2010	Cohort	812	NSAID and aspirin	Filled NSAID/aspirin prescriptions were associated with a reduced risk of oesophageal adenocarcinoma (adjusted incidence density ratio, 0.64; 95%CI: 0.42-0.97) Filled statin prescriptions were associated with a reduction in EAC risk (0.55; 95%CI: 0.36-0.86)	Reduces risk
Corley *et al*[115], 2003	Meta-analysis of 9 studies	1813	NSAID and aspirin	Protective association between any use of aspirin/NSAID and oesophageal adenocarcinoma (OR = 0.57; 95%CI: 0.47-0.71) Intermittent (OR = 0.82; CI: 0.67-0.99) and frequent medication use were protective (OR = 0.54; 95%CI: 0.43-0.67) Any use was protective against both oesophageal adenocarcinoma (OR = 0.67; 95%CI: 0.51-0.87) and squamous cell carcinoma (OR = 0.58; 95%CI: 0.43-0.78)	Reduces risk
Alexandre *et al*[116], 2012	Meta-analysis of 2 studies	1382	Statin	Pooled effect size of 0.53 (95%CI: 0.36-0.78, P = 0.001, I^2 = 0%) for risk of oesophageal adenocarcinoma with prior statin use	Reduces risk
Alexandre *et al*[116], 2012	Meta-analysis of 3 studies	35214	Statin	Pooled effect size of 0.86 (95%CI: 0.78-0.94, P = 0.001, I^2 = 0%) for risk of oesophageal adenocarcinoma wth prior statin use	Reduces risk
Beales *et al*[117], 2012	Case-control	85	Statin	Regular statin use was associated with a significantly lower incidence of oesophageal adenocarcinoma (OR = 0.45, 95%CI: 0.24-0.84) After NSAID/aspirin confounding correction: OR = 0.57, 95%CI: 0.28-0.94	Reduces risk
Heath *et al*[121], 2007	Randomised control trial	100	NSAID (celecoxib)	No difference in the proportion of biopsy samples with dysplasia or cancer between treatment groups in either the low-grade (median change with celecoxib = -0.09); or high-grade (median change with celecoxib = 0.12) stratum	No effect
Singh *et al*[123], 2013	Meta-analysis of 13 studies	9285	Statin	A 28% reduction in the risk of oesophageal adenocarcinoma among patients who took statins (adjusted OR = 0.72; 95%CI: 0.60-0.86)	Reduces risk

NSAID: Non-steroidal anti-inflammatory drug.

Endoscopic resection of specific lesions has been successfully reported (and the resected tissue can be examined by the pathologist). Resection of the entire or circumferential Barrett's mucosa is not recommended due to the risk of stricture formation. It has been reported that complete eradication of high grade dysplasia/early cancer or Barrett's mucosa was achieved in 95% and 89% of patients respectively and the remaining Barrett's mucosa may be treated with ablative therapy[133].

Nevertheless, there are issues around current ablative therapies which include not having an examinable sample; having to wait for the epithelium to regenerate before repeat sampling can take place and the risk of buried dysplastic or neoplastic cells and glands which have the potential to progress undetected. Moreover, there are risks associated with the procedure itself (pain, bleeding, perforation and stricturing), difficulty in interpreting the sampled findings, and undemonstrated long-term outcomes[134].

One of the more novel approaches to ablation involves the use of cryotherapy where tissue is rapidly cooled by liquid nitrogen spray or carbon dioxide gas. Studies demonstrate success rates which are comparable to aforementioned ablative techniques in the treatment of Barrett's oesophagus with high-grade dysplasia (complete eradication of dysplasia in 87%-96% of treated patients and complete eradication of intestinal metaplasia in 57%-96% of treated patients). This success has also been replicated in early-stage oesophageal adenocarcinoma where mucosal cancer was completely eradicated in 75% of patients which included patients that were unsuccessful with other therapies. Cryotherapy is generally tolerated well by patients according to studies but these studies tend to have small sample sizes and short periods of follow-up so the need for more robust studies remains[135].

FUTURE DEVELOPMENTS

As mentioned earlier, many institutions are not able to undertake full Seattle biopsy protocol systematic biopsies. There are adjuncts but their use is limited because they are only used in specialist institutions in the context of research projects and there is a lack of recommendation in the guidelines.

Biomarkers

The endoscopic detection of Barrett's oesophagus and grading of dysplasia are not as reliable as they could be. The need for reliable biomarkers is critical in being able to distinguish Barrett's oesophagus patients who are at risk of developing oesophageal adenocarcinoma[136,137]. The number of publications discussing a potential biomarker for Barrett's oesophagus have increase exponentially over the last 30 years from 1 in 1981 to 1069 in total in 2011 which reflects the fact that Barrett's oesophagus needs a

Table 4 Summary of molecular biomarkers predicting malignant progression

Biomarker	Phase	Sample size	End-point
Biomarker panels			
8-gene methylation panel	3	195	High grade dysplasia/adenocarcinoma
DNA content abnormalities and loss of heterozygosity	4	243	Adenocarcinoma
Expert low grade dysplasia, aneuploidy, *Aspergillus oryzae* lectin	3	380	Adenocarcinoma
DNA content abnormalities			
Aneupolidy/tetraploidy	4	322	Adenocarcinoma
Tumour suppressor loci			
p53 loss of heterozygosity	4	256	Adenocarcinoma
p53 staining	4	48	High grade dysplasia/adenocarcinoma
Epigenetics			
P16 methylation	3	53	HD/adenocarcinoma
Proliferation			
Mcm2	3	27	Adenocarcinoma
Clonal diversity			
Clonal diversity measures	4	239	Adenocarcinoma
Cell cycle markers			
Cyclin A	3	48	High grade dysplasia/adenocarcinoma
Cyclin D1	3	307	Adenocarcinoma
Serum biomarkers			
Leukocyte telomere length	4	300	Adenocarcinoma
Selenoprotein P	4	361	Adenocarcinoma

clinically validated prognostic tool such as an effective biomarker to aid in defining risk.

The Early Detection Research Network has recommended five phases of study before a biomarker can be used clinically[138]. Phase 1 is exploratory to identify markers, phase 2 is for the development of a clinical assay, phase 3 is for retrospective validation, phase 4 is for prospective validation and phase 5 is to test the biomarker on the population with the disease. At present, most biomarkers are in phase 3 and 4. Preclinical studies have been successful in detecting certain biomarkers which contribute to the malignant progression of Barrett's oesophagus but their widespread clinical use is very limited by differences in reproducibility, low sample sizes and the need for multi-centre prospective studies[139-141]. Table 4 is a summary of the biomarkers studied to date[137,142].

Overall, the desire to predict which Barrett's oesophagus patients will progress to oesophageal adenocarcinoma is palpable but remains a target and not a reality. The ideal biomarker as with all potential screening options should be cost-effective, minimally invasive, easily administered and have comparable or superior outcomes to what biopsies currently offer. More work is necessary to ensure that successful biomarkers are smoothly translated into widespread clinical practice.

Metabolomics

Metabolomics is the scientific study of the set of metabolites present within an organism, cell, or tissue and they could play a role in the discovery for a biomarker in Barrett's oesophagus as they are key players in biological systems which are disrupted in disease[143]. A study using urinary metabolomics found that it was possible to separately distinguish Barrett's oesophagus and oesophageal adenocarcinoma from controls as they had different urinary signatures[143]. This suggests that urinary metabolomics and other may have a future role in the pursuit of a non-invasive screening option for Barrett's oesophagus.

Virtual biopsies

Studies have worked on trying to differentiate squamous and columnar epithelia based on their electrical characteristics using electrical impedance *via* a probe[144]. The aim is to reduce discrepancy from inter- and intra-observer variability by having an objective measurement to categorise the epithelium. Magnification endoscopy provides an even more detailed image by optically enlarging the mucosal surface area and studies found that low and high grade dysplasia were consistently identified in Barrett's using this technique but missed using standard endoscopy alone[145]. Confocal laser endomicroscopy (CLE) is a novel technique combining standard white light endoscopy with confocal laser microendoscopy[146]. CLE has demonstrated a high diagnostic value for digestive diseases including Barrett's oesophagus[147-151].

CONCLUSION

Progress has been made in further understanding Barrett's oesophagus since it was first described in 1950. It is a large and increasing health problem with multiple modifiable risk factors, yet there remain several unanswered questions regarding a formal definition, diagnostic criteria, and screening and surveillance needs and methods. Although endoscopy with systematic biopsy and standard pathological examination is currently the mainstay of screening and surveillance for Barrett's oesophagus,

there is still the need for a more cost-effective, less invasive, less cumbersome and more reliable way to conduct diagnosis, screening and surveillance. Primary prevention of Barrett's oesophagus and adenocarcinoma is also of huge interest and potential with studies focussing on the medical treatment of reflux, chemoprevention and anti-reflux surgery.

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REVIEW

Gastrointestinal neuroendocrine peptides/amines in inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) is a chronic recurrent condition whose etiology is unknown, and it includes ulcerative colitis, Crohn's disease, and microscopic colitis. These three diseases differ in clinical manifestations, courses, and prognoses. IBD reduces the patients' quality of life and is an economic burden to both the patients and society. Interactions between the gastrointestinal (GI) neuroendocrine peptides/amines (NEPA) and the immune system are believed to play an important role in the pathophysiology of IBD. Moreover, the interaction between GI NEPA and intestinal microbiota appears to play also a pivotal role in the pathophysiology of IBD. This review summarizes the available data on GI NEPA in IBD, and speculates on their possible role in the pathophysiology and the potential use of this information when developing treatments. GI NEPA serotonin, the neuropeptide Y family, and substance P are proinflammatory, while the chromogranin/secretogranin family, vasoactive intestinal peptide, somatostatin, and ghrelin are anti-inflammatory. Several innate and adaptive immune cells express these NEPA and/or have receptors to them. The GI NEPA are affected in patients with IBD and in animal models of human IBD. The GI NEPA are potentially useful for the diagnosis and follow-up of the activity of IBD, and are candidate targets for treatments of this disease.

Key words: Enteric nervous system; Enteroendocrine cells; Immune cells; Inflammatory bowel disease; Musashi-1; Neurogenin 3; Stem cells

Core tip: Approximately 80% of the body immune cells (IC) are localized in the gastrointestinal (GI) tract close to the GI neuroendocrine regulatory system (NES). Many IC express GI neuroendocrine peptides/amines (NEPA) and possess receptors to several NEPA. Several GI NEPA are abnormal during active inflammatory bowel disease (IBD) in both patients and animal models of IBD. The changes in the GI NEPA are correlated with those of the IC during the inflammatory process. Studying the interactions between the GI NES and the immune system in IBD may improve our understanding of the pathophysiology of IBD and provide us with new tools for treatment.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a lifelong recurrent disorder that comprises three diseases: ulcerative colitis (UC), Crohn's disease (CD), and microscopic colitis (MC). These three diseases have different clinical manifestations, courses, and prognoses[1-3]. Whereas the onset of UC and CD occurs mostly at a young age, MC onset occurs in old age[4,5]. In UC and CD, the activity of the disease varies considerably between patients, from frequent relapses, persistent active disease, to several years of complete remission[4], whereas all MC patients exhibit chronic active disease[6-8]. The inflammation in CD is transmural, in UC it is superficial, and in MC it is in the form of the mucosal and submucosal infiltration of immune cells (IC). CD can arise at any part of the gastrointestinal (GI) tract, while UC and MC affect the recto-colonic mucosa[8,9]. In contrast to UC and CD, spontaneous symptomatic remission occurs in 59%-93% of MC patients[10,11].

IBD diminishes the quality of life considerably and represents an economic problem to both the patients themselves and society[4,9]. The prevalence of IBD amounts to 1.4 million patients in North America and 2.2 million patients in Europe, with 3-20 new cases occurring per 100000 persons annually[12-16]. The prevalence of IBD does not differ among Hispanics, blacks, and Caucasians[17,18]. The incidence of IBD is lower in Asia than in North America and Europe[19-21], but it has been increasing worldwide in recent years[19,21].

The etiology of IBD is not completely understood[9], and the available treatments are not ideal[1-4,22-31]. Typically 70%-80% of the body IC are present in the GI tract in close proximity to the GI neuroendocrine regulatory system (NES)[32,33]. Interactions between the GI neuroendocrine peptides/amines (NEPA) and the immune system have recently been discussed, and it is believed that these interactions play an important part in the pathophysiology of IBD[33-45]. Understanding the role of the GI NEPA in IBD would increase our understanding of the mechanisms underlying the pathophysiology of IBD, and may yield tools for treating these conditions using agonists or antagonists to the GI NEPA[43].

The aim of the present review was to summarize the available data on GI NEA in IBD and to speculate on their possible role in the underlying pathophysiology, and the potential utilization of these peptides/amines in treatments.

GI NES

The NES comprises two parts: the GI endocrine cells in the mucosa and the enteric nervous system (ENS) (Figure 1). The GI endocrine cells occur in all segments of the GI tract except for the esophagus[46,47]. These cells lie between the mucosal epithelial cells facing the GI lumen, and they comprise about 1% of all epithelial cells and produce a large number of hormonal peptides/amines[48-56]. The GI endocrine cells are divided into at least 15 different types depending on the hormone they produce[48,49]. Two hormones can be colocalized in the same type of endocrine cell, such as glucagon-like peptide-1 and glucose-stimulated insulinotropic peptide in the small intestine, and peptide YY (PYY) and oxyntomodulin (enteroglucagon) in the distal small and large intestines[57-60]. It has been shown recently that mature GI endocrine cells can express up to seven different hormones[51,52,61-64].

The GI endocrine cells have specialized sensory microvilli that project into the lumen, and they respond to luminal stimuli (mostly nutrients and/or bacteria byproducts) by releasing their hormones into the lamina propria[32,42,65-85]. The cells also possess a basal cytoplasmic process about 70 μm long that is believed to be involved in their paracrine mode of action[86-90]. It has been shown recently that this process exhibits neuronal axon-like characteristics, and it has been named a neuropod[88,91-93]. The GI endocrine cells also exhibit synaptic vesicles and synthetize presynaptic proteins: synapsin 1, piccolo, bassoon, MUNC13B, RIMS2, latrophilin, and transsynaptic neurexin[88,91-93]. These cells also synthetize transsynaptic neuroligins 2 and 3, homer 3, and postsynaptic density 95[93]. Thus, the GI endocrine cells possess the elements necessary for both afferent and efferent synaptic transmission[93].

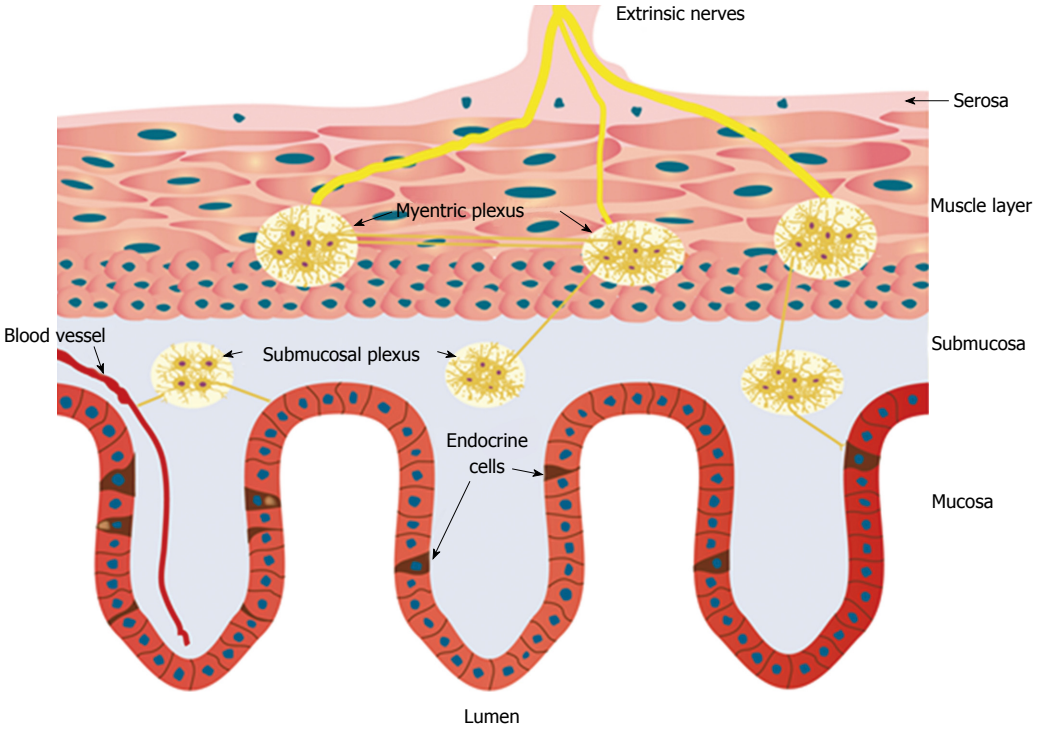

Figure 1 Schematic of the gastrointestinal neuroendocrine regulatory system. The neuroendocrine regulatory system (NES) comprises gastrointestinal (GI) endocrine cells in the mucosa and the enteric nervous system (ENS). The ENS consists of two plexi: one located in the submucosa (the submucosa plexus) and one situated between the longitudinal and circular muscle layers (the myenteric plexus). The GI endocrine cells integrate and interact with each other and with the ENS. The GI NES is an independent system that regulates most of the GI functions and is modulated by the central nervous system.

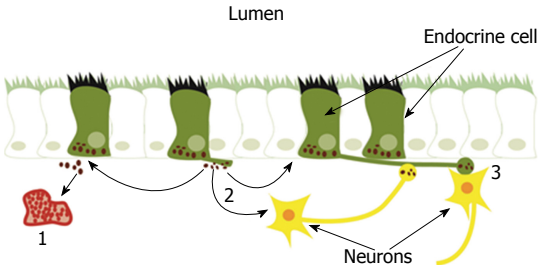

Figure 2 Gastrointestinal endocrine cells have sensory microvilli projecting into the gastrointestinal lumen that register and respond to luminal stimuli by releasing their hormones into the lamina propria. The released hormones exert their effects *via* three modes of action: (1) entering the circulating blood and reaching distant targets (endocrine mode); (2) acting locally on nearby structures (paracrine mode); or (3) *via* synaptic activity. Reproduced from reference 46 with permission from the authors and the publisher.

These data suggest that the GI hormones released in the lamina propria could act locally on close by cells or neurons (paracrine mode), through the circulating blood (endocrine mode), or by afferent and efferent synaptic transmission[94-97] (Figure 2).

Recent observations of GI endocrine cells exhibiting both endocrine and neuron-like characteristics support a long-standing hypothesis about the evolution of the GI NES[98]. The absence of mammalian GI hormonal peptides in the gut of invertebrates, and he occurrence of these peptides in the central nervous system (CNS)[99-101] resulted in the hypothesis that the GI endocrine cells of vertebrates initiated in the nervous system of a common ancestor of invertebrates and vertebrates and then moved during a later stage of evolution into the gut as endocrine cells[98].

The ENS is an independent nervous system within the GI tract that consists of two plexi: one located in the submucosa (the submucosa plexus) and one situated between the longitudinal and circular muscle layers (the myenteric plexus)[102-104]. The neurons of the ENS (about 100 million) are modulated by afferent and efferent nerve fibers from the CNS and the autonomic nervous system[102-104]. The GI endocrine cells integrate and interact with each other and the ENS[105].

The NES regulates GI motility, secretion, absorption, visceral sensitivity, local immune defense, cell proliferation, and appetite[105].

INTERACTION BETWEEN THE GI NES AND INTESTINAL MICROBIOTA

It has long been believed that IBD is caused bacterial infection, and this belief lead to the introduction of salazopyrine (5-aminosalicylic acid-sulfapyridine) for the treat of IBD[106,107]. However, A specific microbe(s) could not be identified as the cause of IBD[106]. Recent studies have shown, however, that intestinal microbiota plays an important role in the pathophysiology of IBD[106]. Thus, low intestinal microbiome diversity and dysbiosis appear to be important factors in the pathophysiology of IBD[106]. The short-chain fatty

acids produced upon fermentation of dietary fibers in the large intestine affect both the immune system and the NES. Butyrate is one of these short-chain fatty acids[108,109]. Butyrate suppresses large intestinal inflammation by inducing T-cell apoptosis, and by suppressing IFN-γ-mediated inflammation[110-112]. The short-chain fatty acids affect several GI peptides, such as PYY and glucagon-like peptide-1[80,113-115]. Furthermore butyrate has been found to affect neurons of the ENS[113,116].

INTERACTIONS BETWEEN THE GI NES AND THE IMMUNE SYSTEM

Several NEPA of the GI NES have been shown to interact with the immune system, including members of the chromogranin/secretogranin family, serotonin, vasoactive intestinal peptide (VIP), members of the neuropeptide Y (NPY) family, substance P, somatostatin, and ghrelin.

Chromogranin/secretogranin family

All of the GI endocrine cell types produce members of the granins family (including chromogranins A and B) that are co-stored and co-released from the GI endocrine cells[34,117-120]. Chromogranin A (CgA) occurs in all GI tract endocrine cell types[121-124]. CgA-derived peptides decrease interleukin (IL)-16 and IL-5 release, and hence decrease the density of lymphocytes at inflammatory sites and thus the proinflammatory action of lymphocytes and monocytes[125-127]. Members of the chromogranin/secretogranin family are believed to exert anti-inflammatory effects.

Serotonin

About 95% of the body serotonin occurs in the GI, of which only 10% occurs in the neurons of the ENS and the rest in the enterochromaffin cells[34,128]. Serotonin is believed to play a pivotal role in intestinal inflammation[34,38,40,125,129,130]. Mast cells, macrophages/monocytes, and T cells are capable of producing serotonin[131]. Serotonin receptors occur in numerous innate IC such as neutrophils, eosinophils, monocytes, macrophages, dendritic cell, mast cells, and natural killer (NK) cells, and in cells of the adaptive immune system such as lymphocytes[130-132]. Serotonin promotes the activation of lymphocytes, whose proliferation protects NK cells and T-helper cells, hinders the apoptosis of IC, and endorses the recruitment of T cells[133-137]. The number of intestinal serotonin cells is decreased in knockout mice lacking T-lymphocyte receptors[125]. Serotonin cells express IL-13 receptors[138]. Against this background, serotonin is considered to be a proinflammatory amine during the inflammatory process.

VIP

VIP is a 28-amino-acid peptide exhibiting structural similarities with secretin[139]. VIP is secreted by neurons, endocrine cells, and IC, and it occurs in almost all body organs[140]. In GI tract, VIP occurs in endocrine cells and neurons of the ENS[141,142]. VIP is believed to be a major immune-regulating neuropeptide that plays an important role in inflammatory disorders, and is considered to be a natural anti-inflammatory agent[142,143].

Both CD4 and CD8T cells produce VIP, especially following antigen stimulation[144,145]. The VIP receptor VPAC1 occurs in lymphocytes, macrophages, monocytes, dendritic cells, microglia, and mast cells[146,147]. VIP inhibits the production of proinflammatory cytokines such as tumor necrosis factor α (TNFα), IL-6, IL-12, iNOS, and promotes the production anti-inflammatory cytokines such as IL-10[148-153]. VIP also inhibits the transcription factors AP-1, nuclear factor-κB (NFκB), CREB, and IRF-1[142,147,153,154], and impairs the acquisition of the macrophage proinflammatory polarization profile[155].

NPY family

The NPY family includes three neuroendocrine peptides that act as hormones and/or neurotransmitters/neuromodulators: NPY, PYY, and pancreatic polypeptide (PP)[156-160]. These peptides consists of 36-amino-acid residues and are structurally related[161]. Whereas NPY is expressed in neurons of the CNS and NES[158,159,162], PYY and PP are expressed by endocrine cells of the ileum, colon, and rectum[163-165]. PP occurs also in endocrine cells in pancreatic islets of Langerhans[160]. NPY and PYY exert similar biological effects[105,161,165], and they act through binding to receptors Y_1 and Y_2[166-169]. T lymphocytes, macrophages, and dendritic cells produce NPY during inflammation[170]. NPY Y_1/Y_2 receptors are localized on IC[171,172], and the binding of NPY to these receptors induces the release of proinflammatory cytokines and nitric oxide from macrophages, neutrophils, and lymphocytes[171,173]. NPY therefore exerts proinflammatory effects in the presence of an inflammatory process. The role of PYY and PP in inflammation is not yet known.

Substance P

Substance P is a member of the tachykinin family and substance P nerve fibers are widely distributed in the GI wall. Substance P is localized in enteric efferent neurons[174-176] and is expressed by several IC including T cells, macrophages, dendritic cells, and eosinophil cells[177-182]. It also plays an important role in the migration of innate IC such as neutrophils and macrophages, and of adaptive IC such as T lymphocytes[183-190]. Furthermore, substance P regulates the proliferation of lymphocytes and modulates the activities of innate and adaptive IC[179,183,191]. Substance P is therefore considered to be one of the main proinflammatory mediators in the GI tract.

Somatostatin

The GI tract and the pancreas contain most of the body

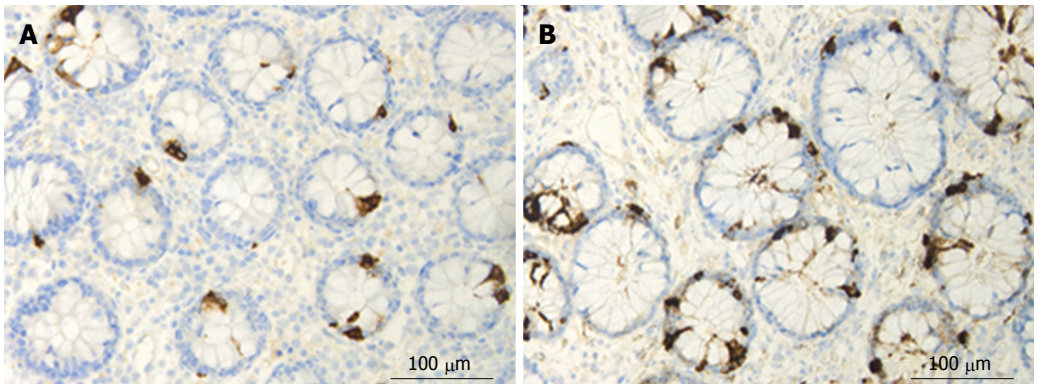

Figure 3 Colonic chromogranin A immunoreactive cells in a control subject (A) and in a patient with lymphocytic colitis (B).

somatostatin[192,193]. About 90% of GI somatostatin is localized in GI endocrine cells, and the remaining 10% is in neurons of the ENS[194]. Somatostatin binds to five membrane G-protein-coupled receptor subtypes (SSTR 1-5)[195]. Several innate and adaptive IC such as monocytes/macrophages, B lymphocytes, T lymphocytes, and dendritic cells expressed these receptors[195-204]. Somatostatin stimulates B-lymphoblast proliferation with the enhancement of immunoglobulin formation[205], inhibits T lymphocytes and granulocyte proliferation, and reduces proinflammatory cytokines such as IFN-g[194,196,206-213]. Somatostatin is considered to be an anti-inflammatory peptide[37,214,215].

Ghrelin

Ghrelin is a peptide composed of 28-amino-acid that occurs mostly in X/A endocrine cells in the oxyntic mucosa of the stomach[42,50,216-221]. Ghrelin performs several functions, including controlling food intake, energy homeostasis, and GI motility[217,218,221-224]. It also mediates the immune response and inflammation[146,225-227]. The anti-inflammatory prosperities of ghrelin are due to it modulating the secretion of pro- and anti-inflammatory cytokines from LPS-stimulated macrophages[225].

NES NEPA IN IBD

NES abnormalities in IBD

Changes in the ENS in IBD such as an increase in the number of enteric neurons, and altered neurotransmitter synthesis and release have been described[228-237]. Similarly, the density of the GI endocrine cells, the proportions of different endocrine cell types, and the release of GI NEPA are affected in both IBD patients and animal models of human IBD.

Chromogranin/secretogranin family: The circulating level of CgA is elevated in IBD patients and is reduced following treatment with certain biological agents[56,238-241]. Patients with IBD exhibit elevated concentrations of fecal CgA and secretogranins[242,243]. The CgA cell density is increased in patients with IBD, and in animal models of human UC and CD, with the exception of trinitrobenzene sulfonic acid (TNBS)-induced colitis[9,117,244-248] (Figure 3). The administration of the proinflammatory cytokines INFγ and TNFα and the induction of colitis by dextran sodium sulfate (DSS) in mice were found to increase the number of CgA cells[249].

Serotonin: The density of colonic serotonin cells is elevated in patients with UC, CD, and lymphocytic colitis[117,250] (Figure 4). The serotonin cell density was also increased in an animal model of human UC (TNBS-induced colitis in rats) and in an animal model of human CD (DSS-induced colitis in rats), as well as in other animal models of human UC and CD, and in IL-2-knockout mice[230,244,245,251,252].

VIP: Studies of VIP in patients with IBD have produced conflicting results. The immunohistochemical examination and quantification of tissue extracts from rectal biopsy samples obtained from patients with UC and CD showed an increased number of VIP-positive nerve fibers and an increased VIP concentration in CD but not in UC[253]. Other studies found that the number of VIP-positive nerve fibers was either decreased or unchanged in patients with UC and CD[245,254,255]. These contradictory results for VIP in patients with IBD could be explained by VIP occurring mostly in neurons of the ENS and that analyzing VIP in small mucosal biopsy specimens obtained during an endoscopic examination does not produce reliable results. However, changes in VIP have been found in animal models of human IBD, especially knockout mice[251]. In *IL-2* gene-knockout mice, the relative volume density of VIP-positive nerve fibers and the level of VIP in tissue extracts were both decreased[251].

NPY family: The density of NPY enteric neurons increased as well as hyperplasia of NPY nerve fibers have been observed in mice with colitis induced either by DSS or streptomycin-pretreated *Salmonella typhimurium*[256,257]. The PYY cell density is increased in patients with UC and lymphocytic colitis as well

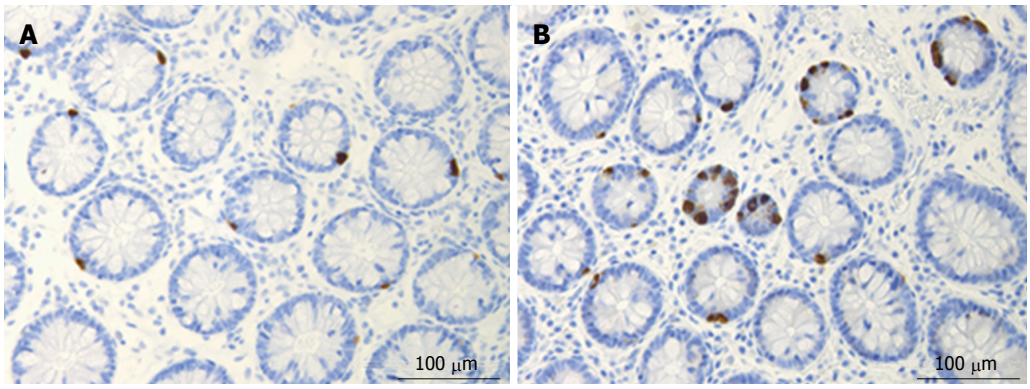

Figure 4 Colonic serotonin cells in a control subject (A) and in a patient with lymphocytic colitis (B).

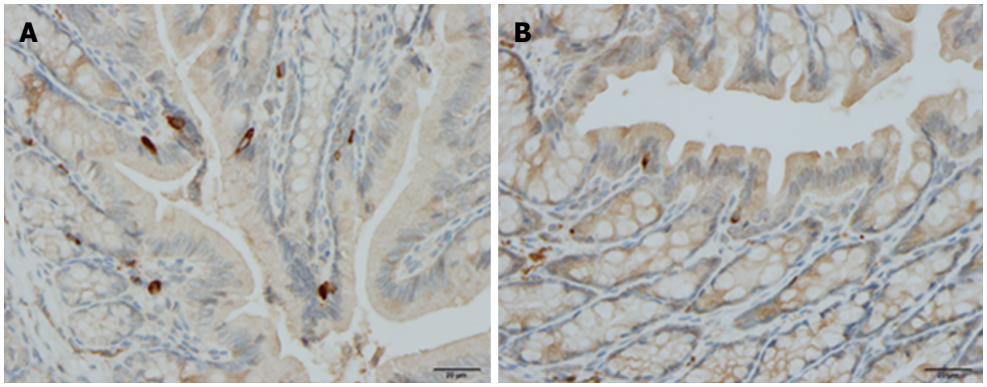

Figure 5 Colonic somatostatin immunoreactive cells in a control rat (A) and in a rat with dextran sulfate sodium-induced colitis (B).

as in colitis induced by DSS in rats and in *IL-2* gene-knockout mice[117,245,250,251]. The PYY cell density is decreased in CD, with this change being correlated positively with the increased disease severity[117]. Similarly, the density of PYY cells was reduced in an animal model of human CD, namely TNBS-induced colitis in rats[244]. The robust positive correlation between the PYY cells and IC found in colitis induced either by DSS, or TNBS in rats is suggestive of an interaction between PYY cells and the IC[244,245]. It is noteworthy that PYY and oxyntomodulin (enteroglucagon) are produced from the same endocrine cell (L cells)[57]. Whereas the density of oxyntomodulin-containing cells is increased in patients with CD and in both DSS- and TNBS-induced colitis, and in IL-2 gene-knockout mice, it is unchanged in patients with UC[118,244,251,258]. The PP cell density is decreased in patients with CD and in colitis induced by either DSS, or TNBS in rats[117,245,248].

Substance P: The levels of substance P are increased in tissue extracts from the colon and in the rectum of patients with UC and CD, and were correlated with disease activity[253,259-261]. The density of nerve fibers immunoreactive to substance P is decreased in the colon of UC patients[262]. The density of substance-P-immunoreactive fibers has been reported to be both increased[253,262] and unchanged[262] in the colon of CD patients. The concentration of substance P in the colon of IL-2-knockout mice is decreased, while substance-P-immunoreactive cells were unchanged[251].

Somatostatin: The number of somatostatin cells is decreased in the colon of patients with IBD, and in animal models of human IBD, except for TNBS-induced colitis where it is increased[245,263-265] (Figure 5).

Ghrelin: The circulating levels of ghrelin are elevated in patients with IBD with active inflammation[266,267]. Moreover, circulating ghrelin levels in UC and CD patients are correlated with TNFα, C-reactive protein, the erythrocyte sedimentation rate, and fibrinogen, and negatively correlated with nutritional status parameters[42,228,268,269].

Possible mechanisms underlying NES abnormalities in IBD

The mechanisms underlying the changes in ENS during inflammation in IBD remain unclear. However, recent studies have shed some light on the possible mechanisms of the inflammation-induced changes in the GI endocrine cells in IBD[258,270].

Whereas changes in GI endocrine cells do occur in UC, CD, lymphocytic colitis, and animal models of human IBD, the nature of these changes differ between the different IBDs and animal models

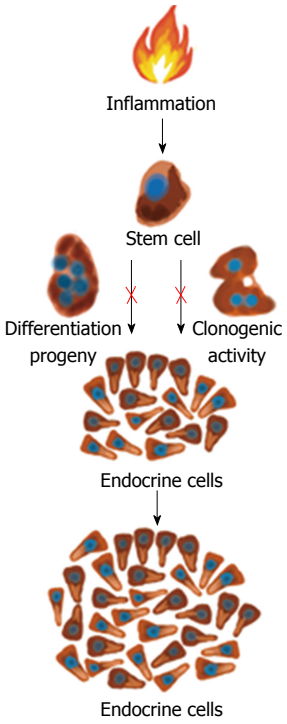

Figure 6 Proinflammatory substances such as cytokines may act on the intestinal stem cells and increase their clonogenic and differentiation progeny so that the density of intestinal endocrine cells increases during active inflammation.

of human IBD[9,117,244-248,250,259]. The changes in GI endocrine cells can be explained by two different mechanisms: abnormal stem cell clonogenic and differentiation progeny toward endocrine cells activities (Figure 6), and switching on and off of the expression of certain GI NEPA (Figure 7).

Abnormal stem cell clonogenic and differentiation activities: Each intestinal crypt contains four to six stem cells that either divide into identical new stem cells (clonogenic) or differentiate into all types of epithelial cells through a series of progenitors[270-281]. This differentiation into epithelial cells includes the secretory and absorptive lineages. The secretory lineage gives rise to endocrine, goblet, and Paneth cells. The absorptive lineage results to absorptive enterocytes[270-281]. In rats with TNBS-induced colitis, which is an animal model of human CD, the colonic density of Musashi-1 (Musi-1) immunoreactive cells was found to be reduced[258]. In contrast, the colonic density of Musi-1 cells was unaffected in rats with DSS-induced colitis, which is an animal model of human UC[268]. Musi-1 is located in both intestinal stem cells and early progenitors[282-284]. These observations indicate that the clonogenic activity of stem cells is affected in an animal model of CD but not in one of UC. This is probably due to the inflammation associated with CD being deep while that associated with UC being superficial.

In rats with both TNBS- and DSS-induced colitis, the colonic Math-1 cell density was found to be unaffected. Math-1 occurs early progenitor in the secretory lineage, and mutant (Math-$1^{-/-}$) mice have no secretory cells[285].

The colonic neurogenin 3 (Neurog3) cell density is reduced in rats with TNBS-induced colitis, while it is increased in rats with DSS-induced colitis[259,270]. Neurog3 is localized in an early progenitor belonging to the secretory lineage, which contributes to the differentiation into endocrine cells[286]. Transgenic mice (Neurog$3^{-/-}$) do not have enteroendocrine cells, but normal densities of goblet and Paneth cells[286-288]. Similar to Neurog3, the colonic NeuroD1 cell density is decreased in rats with TNBS-induced colitis while it is increased in rats with DSS-induced colitis[269,282]. NeuroD1 is located in progenitors originated from Neurog3 progenitors[289,290]. Mice deficient in NeuroD1 lacks certain types of enteroendocrine cells[53,291]. These findings show that the differentiation progeny toward endocrine cells is affected in animal models of human IBD.

Switching the expression of NEPA on and off: As mentioned above, mature GI endocrine cells can express up to seven different hormones[51,52,61-64]. It seems that the changes in the proportion of GI endocrine cells during inflammation occur *via* switching off the synthesis of a neuroendocrine peptide/amine and switching on the synthesis of another[270]. Such a phenomenon has been reported in rats with TNBS-induced colitis (Figure 8).

Hypothesis: It may be speculated that during the inflammation that occurs in active IBD, the IC produce proinflammatory cytokines and other substances that affect the GI stem cells and mature endocrine cells. This will induce abnormal clonogenic and differentiation activities of stem cells. Moreover, the mature endocrine cells switch off the expression of a certain hormone in favor of switching on the synthesis of another hormone. This would result in changes in the total density of endocrine cells and in the proportion of different endocrine cell types. NPA produced by the altered endocrine cells would in return affect the IC *via* their NPA receptors (Figure 9).

CLINICAL IMPLICATIONS

The changes in the intestinal NES associated with inflammation in IBD patients are believed to be useful tools for the diagnosis and follow-up of disease activity. Furthermore, the GI NEA could be candidate targets of IBD treatments. Thus, agonists to anti-inflammatory NEPA and antagonists to proinflammatory NEPA can be used not only for their pharmacological effects but also to correct a pre-existing imbalance in GI NEPA caused by inflammation.

Diagnosis

The colonic CgA cell density has been shown to be a

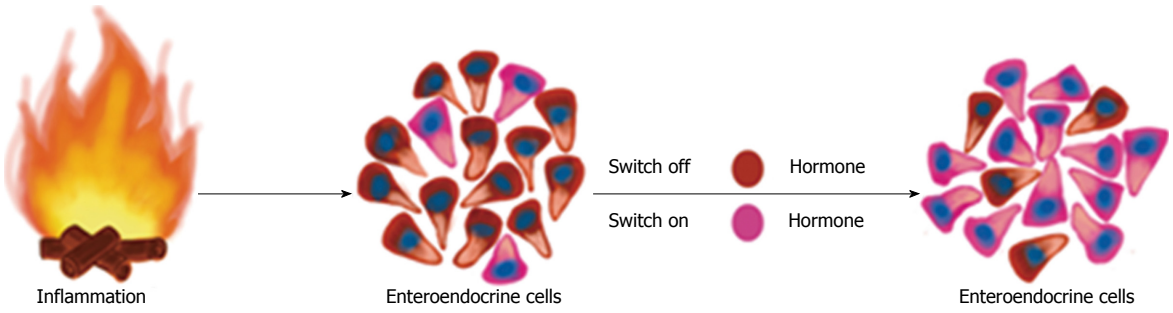

Figure 7 During the inflammatory process, several proinflammatory substances may act on the mature endocrine cells so that they switch off the expression of a certain neuroendocrine peptides/amines and switch on the expression of another neuroendocrine peptides/amines.

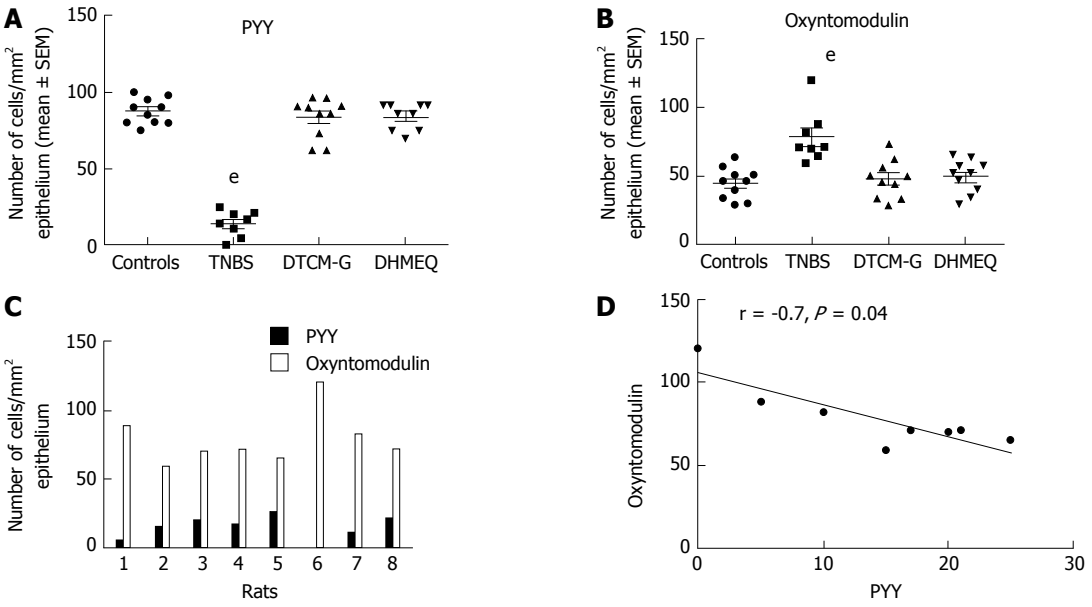

Figure 8 Colonic densities of (A) peptide YY-positive cells and (B) oxyntomodulin (enteroglucagon)-positive cells in control rats, in rats with trinitrobenzene sulfonic acid (TNBS)-induced colitis, and in rats with TNBS-induced colitis treated with 3-[(dodecylthiocarbonyl)-methyl]-glutarimide (DTCM-G, an activator protein-1 inhibitor) and dehydroxymethylepoxyquinomicin (DHMEQ, a nuclear factor-κB inhibitor). Densities of PYY-positive and oxyntomodulin-positive cells in each rat of the TNBS group (C), and their correlation (D). $^{e}P < 0.001$ *vs* controls. Reproduced from reference 274 with permission from the authors and the publisher. PYY: Peptide YY.

good biomarker for diagnosing lymphocytic colitis, with a high sensitivity and specificity[9]. The blood and fecal levels of CgA and secretogranins have been proposed for the diagnosis and follow-up of the disease activity in IBD[56,238-243].

Treatment

Treatment with CgA-derived peptides of mice with DSS-induced colitis decreases the disease activity index, macroscopic and histology scores, and the colonic levels of IL-1β, IL-6, and TNFα[34].

Antagonists of serotonin receptors 5-HTR3 and 5-HTR7 such as tropisetron, granisetron, ondansetron, ramosetron, and SB-269970 have shown anti inflammatory effects in animal models of human IBD[292-300]. These serotonin receptor antagonists act *via* reducing the ynthesis of proinflammatory cytokines IL-1, IL-6, and TNFα. The usefulness of selective inhibition of mucosal serotonin by these receptor antagonists in the clinical treatment of IBD remains to be determined[301].

VIP is believed to be a potential agent for treating IBD since it targets both the innate and adaptive immune responses and inhibits the secretion of numerous proinflammatory cytokines *via* its actions on AP-1 and NFκB[142]. Administering VIP reduced inflammation in TNBS-induced colitis in mice[142], and it has been used successfully in the clinic as an inhalator for treating pulmonary hypertension and sarcoidosis[142]. However, delivering VIP is problematic since it is degraded rapidly in the blood circulation (with a half-life of only 1-2 min) and systemic administration causes both cardiovascular and intestinal side effects[140,302,303].

NPY occupies a key position during the inflammatory process in IBD, and NPY antagonists could be potentially useful in treatments for the inflammation in IBD[43]. This suggestion is supported by observations made in animal models of UC, namely DSS-induced colitis in rats[303,304]. Treatment with NPY

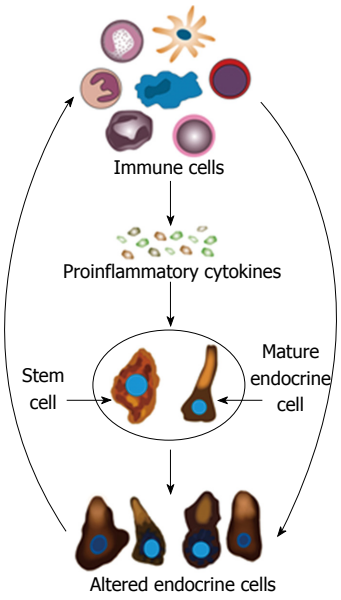

Figure 9 Schematic of the possible mechanisms underlying the changes in gastrointestinal endocrine cells in inflammatory bowel disease. In active inflammatory bowel disease, the immune cells (IC) produce proinflammatory cytokines and other substances that affect the gastrointestinal (GI) stem cells and mature endocrine cells. Thus, abnormal clonogenic and differentiation activities are induced in stem cells. Moreover, the mature endocrine cells switch off the expression of certain hormones in favor of switching on the synthesis of other hormones. This would change the GI endocrine cell density and alter the proportions of the various endocrine cell types. The NPA released by the altered endocrine cells would in return affect the IC *via* their NPA receptors.

antisense oligodeoxy-nucleotides in colitis induced by DSS in rats reduced the inflammation as well the concentration of NPY, TNFα, p-Akt, and asp-NFκB[304]. The NPY Y_1 receptor is involved in several biological functions[302-305], and so using an NPY Y_2 receptor antagonist is preferable in future clinical implications in order to minimize side effects.

Blocking substance P receptors with either substance P antibodies or with CP 96345 (NK-1R antagonist) diminished jejunal inflammation[306,307].

The effects of ghrelin treatment were tested in an animal model of human UC, namely TNBS-induced colitis in mice[147,307]. Ghrelin decreased both the clinical and histopathological severity of the colitis and increased the survival rate[147,307]. These effects seem to be attributable to the decrease of both inflammatory and Th1-driven autoimmune responses *via* affecting several inflammatory mediators, and by the involvement of IL-10/transforming growth factor-ß1-secreting regulatory T cells[147,307].

CONCLUSION

IBD is a chronic disease with unknown etiology that affects a large number of individuals worldwide. About 80% of the body IC are in the GI tract close to the NES of the gut. Several innate and adaptive IC express and release a considerable number of GI NEPA. Furthermore, the IC possess receptors for several GI NEPA. The enteroendocrine cells and the neurons of the ENS are abnormal during the inflammation that occurs in IBD. The changes in these two compartments of the GI NES are strongly correlated with the changes in IC in active IBD. These observations indicate the presence of interactions between GI NEPA and the immune system in active IBD. These interactions seem to play a significant role in the pathophysiology of IBD. The changes in the GI NEPA during active IBD occur in proinflammatory GI NEPA such as serotonin, members of the NPY family, and substance P, and in anti-inflammatory GI NEPA such as members of the chromogranin/secretogranin family, VIP, somatostatin, and ghrelin. Antagonists to the proinflammatory GI NEPA and agonists to the anti-inflammatory GI NEPA could therefore be useful tools for treating IBD.

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REVIEW

Colorectal cancer screening: An updated review of the available options

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Abstract

Colorectal cancer (CRC) is a significant cause of morbidity and mortality worldwide. However, colon cancer incidence and mortality is declining over the past decade owing to adoption of effective screening programs. Nevertheless, in some parts of the world, CRC incidence and mortality remain on the rise, likely due to factors including "westernized" diet, lifestyle, and lack of health-care infrastructure and resources. Participation and adherence to different national screening programs remain obstacles limiting the achievement of screening goals. Different modalities are available ranging from stool based tests to radiology and endoscopy with varying sensitivity and specificity. However, the availability of these tests is limited to areas with high economic resources. Recently, FDA approved a blood-based test (Epi procolon®) for CRC screening. This blood based test may serve to increase the participation and adherence rates. Hence, leading to increase in colon cancer detection and prevention. This article will discuss various CRC screening tests with a particular focus on the data regarding the new approved blood test. Finally, we will propose an algorithm for a simple cost-effective CRC screening program.

Key words: Colorectal cancer; Colonoscopy; Occult blood; Fecal DNA test; Blood DNA test; Screening; Guidelines

Core tip: Multiple societies have published screening guidelines concerning colorectal cancer (CRC) screening. Despite that, global participation can be challenging due to wide variability in the availability of screening tools, especially the newer resources. Additionally, patient friendly approach, improving patient uptake, adherence, and compliance to attain national CRC screening goals are still lacking. Regardless of the screening approaches utilized, it is necessary to demonstrate high sensitivity for detection of advanced neoplasia and CRC, as well as high specificity for costeffectiveness. Furthermore,

they must have broad acceptability to the general population, healthcare providers, and third-party payers. Hence, achieving most of the screening value that is derived from cancer prevention over cancer detection. Recently, a very appealing blood test was FDA approved for screening, this modality certainly carries significant appeal, but how does it fare when we compare to the rest of tests? This is the question we aim to answer through this review.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and the second most common cancer in women worldwide[1] with 1.36 million people affected globally, accounting for nearly 10% of cancers[2]. It remains the second leading cause of cancer in the United States and worldwide[1,3]. Due to its slow progression from detectable precancerous lesions and to the much better prognosis of patients diagnosed at early stages, the potential for reducing the burden of the disease by early detection is significant. While screening has noticeably been shown to reduce the risk of CRC associated mortality[4], its effectiveness is jeopardized by a multitude of factors including the limitations of test performance, lack of accessibility, and suboptimal screening compliance. Consequently, resulting in a marked variation in CRC incidence and mortality globally[1,5]. The newly approved blood screening test overcomes most of the above mentioned factors, this may lead to better participation rates. This article will discuss various CRC screening tests particularly the new blood based test. Moreover, we will debate the different CRC risk assessment scores, and screening programs participation and adherence rates, as well as the issue of total cost. Furthermore, we will propose a potential screening algorithm that might attain a high rate of population participation and adherence, especially in lower income countries.

SCREENING TESTS

The ideal screening study should be efficient with high sensitivity and specificity, safe, available, convenient, and cheap. Current CRC screening methods are divided into invasive and non-invasive tests.

The non-invasive tests include stool and blood-based tests and radiologic tests. The stool-based tests currently available are the guaiac-based fecal occult blood test (gFOBT), fecal immunochemical test (FIT), and the newer fecal DNA testing (Multitarget stool DNA, MT-sDNA, Cologuard®). Those tests are based on the concept of detecting blood or shredded cell debris by vascularized polyps, adenomas and cancers[6]. The radiologic examinations include double contrast barium enema, capsule endoscopy and Computed tomographic colonography (CTC). Their role revolves around radiographic visualization and identification of an advanced colonic polyp or cancer in addition to the possibility of detection of extra-colonic findings (by CTC). The newly emerged blood test (Epi procolon®) is a qualitative *in vitro* diagnostic polymerase chain reaction (PCR) test for the detection of mutated methylated septin9 DNA in EDTA plasma derived from patient whole blood specimens. Methylated SEPT9 has been associated with the occurrence of CRC[7].

Invasive tests include the flexible sigmoidoscopy (FS) and colonoscopy which offer direct visualization and detection of a colonic polyp or advanced neoplasia with the advantage of getting a pathology specimen. In real life, colonoscopy has been introduced as a primary screening tool in a number of countries in recent years including United States, Germany, Poland, Austria, and parts of Italy. gFOBT is still being used in France, Finland, and the United Kingdom. Netherlands and many other European countries have shifted from gFOBT to FIT. Sigmoidoscopy remains a screening option in the United Kingdom. In United States all the available screening methods are still being offered.

SCREENING GUIDELINES

In the United States, the two main principle guidelines for CRC screening are: (1) joint guidelines from the American Cancer Society, the United States MultiSociety Task Force on Colorectal Cancer, and the American College of Radiology[8]; and (2) the United States Preventive Services Task Force guidelines which have been updated recently[9]. Other organizations have issued their own guidelines as well, such as the American College of Gastroenterology[10], the American College of Physicians[11], and the National Comprehensive Cancer Network[12]. Table 1 summarizes the latest recommendations from these different sets of guidelines for average-risk individuals. It is noteworthy that almost all the guidelines are coherent in terms of their recommendations, with the exception of barium enema being dropped for reasons of low sensitivity of only 48%[13]. Additionally, the frequency of stool DNA analysis is controversial, as such a test is rather new. Nonetheless, all the remaining screening options are well endorsed by all the societies as potential screening methods.

CRC SCREENING HISTORY, INCIDENCE, AND MORTALITY

In the 1980's and 1990's, most screening methods put in use were FOBT and sigmoidoscopy. However, since the year 2000, most CRC screening in the United States

Table 1 Updated guidelines of the various societies regarding colorectal cancer screening

	ACG (2009)	ACP (2015)	NCCN (2015)	USPSTF (2016)	ACS (2016)
Sigmoidoscopy (yr)	Q 5-10	Q 5	Q 5	Q 5 or Q 10 + stl	Q 5
Colonoscopy (yr)	Q 10	Q 10	Q 10	Q 10	Q 10
CT colonography (yr)	Q 5	N/A	N/A	Q 5	Q 5
Ba enema (yr)	N/A	N/A	Q 5	N/A	Q 5
Stool eFOBT (yr)	Q 1	Q 1	Q 1	Q 1	Q 1
Stool FIT (yr)	Q 1	Q 1	Q 1	Q 1	Q 1
Stool MT-sDNA (yr)	N/A	N/A	N/A	Q 1-3	Q 3

FIT: Fecal immunochemical test; CT: Computed tomography.

shifted towards colonoscopy, even though its effects on reducing CRC incidence and mortality has never been proven in a solid randomized controlled trail. Therefore, the evident question would be: has it been effective?

Data from a recent study examining the incidence of CRC in males and females established a drop in both incidence and mortality from CRC in both genders concomitant with the beginning of the screening programs[3]. In addition to that, over a 20-year period (SEER data 1991-2011), the United States colorectal cancer incidence (all races and genders confounded) has declined from 59.5 cases to 39.3 cases per 100000 (35% reduction) with a corresponding mortality reduction over the same time period from 24.0 to 15.1 deaths per 100000 (37% reduction)[14]. Moreover, in the National Polyp Study (NPS), CRC was prevented by colonoscopic removal of adenomatous polyps. They evaluated the long-term effect of polypectomy in a study on mortality from CRC. Among 2602 patients who had adenomas removed during participation, after a median of 15.8 years, 1246 patients had died from any cause and 12 had died from CRC. Given an estimated 25.4 expected deaths from CRC in the general population (SEER cohort), the standardized incidence-based mortality ratio was 0.47 (95%CI: 0.26-0.80) with colonoscopic polypectomy, suggesting a 53% reduction in mortality[15]. Although the NPS does not address the effectiveness of screening colonoscopy in the general population, their findings provide an indirect estimate of the effect of removing adenomas, which is the primary interventional measure in screening colonoscopy. These findings support the hypothesis that colonoscopic removal of adenomatous polyps prevents death from CRC.

SCREENING OPTIONS

FS and colonoscopy

Colonoscopy is considered to be the gold standard tool of screening with a high sensitivity and specificity. This test affords the opportunity to detect and resect neoplasia and precancerous lesions across the entire large bowel and is the definitive examination when other screening tests are positive. It is relatively safe with recent data suggesting a less than 1/1000 perforation rate most often due to a polypectomy rather than the act itself. On the other hand, colonoscopy requires full bowel preparation and sedation[16-20]. Moreover, despite it being readily available, it is not considered cheap or easily affordable to the general population, hence rendering its application difficult on mass screening basis[21-24]. Multiple case-control and prospective cohort studies have estimated cancer mortality to be 68% to 88% lower among persons who undergo screening colonoscopy than among those who do not[18,24-26]. A meta-analysis of observational studies showed that despite a 68% lower overall mortality, limited benefit from colonoscopy was seen with respect to cancer in the proximal colon[17]. Another study showed that there was a 29% reduction in overall CRC mortality, a 47% reduction in mortality from distal CRC, and no reduction in mortality from proximal CRC. This study concluded that colonoscopy significantly reduces mortality from CRC, but the benefit is not uniform across different areas of the colon[22]. This discrepancy may be due to several factors affecting the quality of the act itself[27-31] (*i.e.*, incomplete colonoscopy, training level and experience of the gastroenterologist, inadequate bowel preparation, or technical difficulties with polyp removal in the proximal colon) or possibly differences in the biologic characteristics of proximal and distal colorectal cancers[32]. To address these issues thoroughly, data from large controlled randomized trails are still lacking but are currently under way. The Colonoscopy *vs* Fecal Immunochemical Test in Reducing Mortality from Colorectal Cancer (CONFIRM) trial (ClinicalTrials.gov number, NCT01239082) is a randomized comparison of one-time colonoscopy with annual FIT plus colonoscopy as follow-up to a positive test, to examine CRC incidence and mortality over 10 years. A similar trial comparing colonoscopy with FIT is being conducted in Spain (COLONPREV) trail (ClinicalTrials.gov number, NCT00906997)[33]. Two additional European studies are comparing screening colonoscopy with no screening [the Nordic-European Initiative on Colorectal Cancer (NordICC)] trial (ClinicalTrials.gov number, NCT00883792)[34,35] or with FIT or no screening [Screening of Swedish Colons (SCREESCO), NCT02078804] with respect to mortality from CRC.

Sigmoidoscopy offers limited bowel preparation compared to colonoscopy. In addition, several randomized controlled trials have shown that screening with FS, followed by colonoscopy if precancerous polyps

are detected, reduces CRC mortality[36,37]. Analysis from several large, randomized, controlled trials have confirmed the efficacy of one-time and periodic (every 3 to 5 years) sigmoidoscopy, with a 26% to 31% lower mortality from CRC among patients who underwent FS screening than among those who underwent no screening[17,38-41]. However, the benefit of sigmoidoscopy is limited to cancer in the distal colon (rectum, sigmoid, and descending colon), for which the reduction in mortality was reported to be 46%[17]. Many programs have abandoned this strategy in favor of colonoscopy for better prevention results.

gFOBT

gFOBT detects the presence of blood in feces through a chemical reaction dependent upon the peroxidase activity of heme. It is an inexpensive, simple, and widely available test. A landmark study that evaluated the fecal occultblood test randomized 46551 participants 50 to 80 years of age to screening for CRC on once a year basis, every two years, or to a control group. They concluded that annual fecal occult-blood testing with rehydration of the samples decreased the 13-year cumulative mortality from CRC by 33%[42]. Another randomized study compared mortality rates after FOB tests every 2 years during a 10-year period with those of unscreened similar controls. They found that after 10 years of follow-up, screening by FOB every 2 years (Hemoccult-II without rehydration) led to a reduction of 18% in CRC mortality. This was independent of sex and age, in individuals aged 45-75 years[43,44]. In the Minnesota Colon Cancer Control Study a 30-year follow-up of patients randomly assigned to annual/or biennial gFOBT *vs* usual care showed a 32% decrease in CRC mortality. Incidentally mortality reduction was more pronounced in men compared to women[45]. Several other randomized, controlled trials have shown lower mortality from CRC with this strategy compared to no screening[46-49]. However, this test requires a moderate quantity of heme to effect a visible change in color and thus is not analytically very sensitive to the presence of blood[6]. Once-only test sensitivity for cancer may approximate 50%[50] although many other studies indicate it is lower[51]. The method relies on simple oxidation, and therefore, any dietary peroxidases, such as heme from myoglobin in red meat, peroxidase in plants, *etc.*, or any antioxidant, such as vitamin C, have the potential to confound the result. The gFOBT is therefore an inherently non-specific test with a very low PPV of 3%-10%[8,52]. Consequently, a test showing higher statistical results was urgently needed.

FIT

FIT is considered to be a newer version of the guaiac based FOBT. It is an antibody to human globin that does not cross react with dietary meats. Therefore, no need to avoid foods with peroxidase activity. FIT carefully measures the colonic blood since upper gastrointestinal globin is degraded readily by digestive proteolytic enzymes. The FIT sampling technique is simple and easy to collect with fewer fecal samples required compared to FOBT. Trials have shown that FIT has a greater sensitivity for detecting advanced adenomas and CRC than both standard and sensitive gFOBT[53-57]. More recent systematic review and meta-analysis including 19 qualified studies showed an overall accuracy of FIT for detection of CRC of 95% with a cumulative respective 79% sensitivity and 94% specificity[58]. Moreover, data for its protective effect can already be extrapolated from several screening programs. For instance, an organized biennial single FIT screening program in Florence where 6961 were screened with an average follow-up period of 11 years have shown a 22% reduction in CRC incidence[59]. One of the disadvantages of FIT is its low sensitivity for detecting colon polyps[57]. Additionally, many types of tests are available, the measures of accuracy vary greatly between tests within a technology as well as between technologies and according to how the test is applied (*e.g.*, sample number) and the various confusing cutoff levels[60-62].

CTC

CTC or virtual colonoscopy is a rapid radiographic non-invasive imaging test that requires no sedation with lower procedural risks compared to colonoscopy[63-65]. In addition to that, it carries the advantage of extra colonic evaluation[66-69]. In a recent comparative meta-analysis the estimated pooled sensitivity and specificity per patient for polyps detection in asymptomatic screened patients were 66.8% and 80.3% respectively for CT colonography, and 92.5% and 73.2% respectively for colonoscopy. Analysis according to size showed that both studies have similar sensitivity for large polyps but evident lower CTC sensitivity for polyps < 8 mm. Regarding overall detection of CRC, the pooled sensitivity of CT colonography (96%) was not statistically significant from that of colonoscopy (91%)[70]. However, CTC is not a very pleasant study since the patient must take the same preparation as for colonoscopy in addition to the same discomfort during procedure insufflation. Moreover, contrast allergy, radiation exposure, and the need for colonoscopy if positive findings are considered additional disadvantages of CTC[71]. Perforation risk is an existing drawback, although to a lesser degree than colonoscopy[72]. To note that, published data from randomized studies evaluating the impact of CT colonography on CRC incidence and mortality are lacking.

Stool DNA testing

In August 2014, Cologuard® became the first multi-target stool DNA approved by the FDA for general CRC screening[73]. Stool DNA test targets molecular debris in stool including abnormal DNA present in malignancies such as mutant KRA, actin, FIT, aberrantly methylated

BMP3, and NDRG4 promoter regions. One multicenter study on nearly 10000 patients comparing Cologuard® to FIT using colonoscopy as the gold standard showed that the fecal DNA test had a higher sensitivity than FIT for detecting CRC (92% *vs* 74%). Unfortunately, Cologuard® detected fewer than half of all large advanced adenomas (42%), limiting its preventive role. Fecal DNA test had lower specificity at 87%-90% compared to FIT (95%-96%)[74]. Moreover, a new elegant study from Stanford university used a Markov model of average-risk CRC screening to compare the effectiveness and cost effectiveness of screening with the MT-sDNA test *vs* FIT or colonoscopy. They found FIT and colonoscopy to be more effective and less costly than the MT-sDNA test when participation rates were equal for all strategies. For the MT-sDNA test to be cost effective, the patient support program included in its cost would need to achieve substantially higher participation rates than those for FIT, whether in organized programs or under the opportunistic screening setting that is more common in the United States[75]. Additionally, the screening interval differs between FIT and MT-sDNA, which makes a comparison of the effectiveness of any programed screening difficult.

Methylated SEPT9

Septins are a group of scaffolding proteins that provide structural support during cell division[76]. Individual septins exist in stable six-to eight-subunit core heteromers, and the octamer contains two molecules of each of SEPT2, SEPT6, SEPT7, and SEPT9 subunits[77]. It was suggested that SEPT9 occupies a terminal position in the complex and plays a key role in subunit polymerization and the whole octamer stabilization[78]. It is also critical for the final separation of daughter cells during cytokinesis[79]. Therefore, cytokinesis may be seriously affected if abnormal SEPT9 or no SEPT9 is expressed, and this could be a key factor in CRC carcinogenesis when the promoter region of the SEPT9 gene is hypermethylated and the transcription is compromised[80]. Hypermethylated Septin9 DNA can be found in the tumor DNA that has been shed into the bloodstream from all intestinal anatomical sites[81]. Epi proColon® (also referred to as the mSEPT9 assay) became FDA approved for CRC screening in April 2016, it is the first blood test used for this goal. The mSEPT9 assay relies on qualitative detection by Real-Time PCR of the methylated Septin 9 gene that is present in increased levels in patients with colon cancer[82,83] (Figure 1). In initial retrospective case-control studies, the mSEPT9 test showed a great promise, with a sensitivity of about 70% and specificity of 90% for CRC detection[81,82]. A subsequent prospective trial in an asymptomatic screening cohort reported lower rates of sensitivity (48%) and specificity (92%) for CRC. However, this sensitivity decreased to 35% for stage I CRC and 11% for advanced adenomas almost totally eliminating its preventive role[84]. In a prospective multi-center study, compared with FIT, Sept9 testing showed similar sensitivity (68% *vs* 73%) but markedly decreased specificity (97% *vs* 81%). While the overall sensitivity for CRC detection of Septin9 may be superior to gFOBT, it is non-inferior to that of FIT[85]. However, relative to Cologuard®, the Epi proColon® test appears to be less sensitive for both CRC and advanced adenomas in actual practice, but with a higher specificity for cancer[86].

Nonetheless, evidence suggests that some patients who are reluctant to undergo the usual screening would be receptive to a blood test. An observational study showed that 97% of subjects refusing colonoscopy accepted a non-invasive screening test, of these 83% chose a blood test. This demonstrates that offering non-invasive test options might significantly increase compliance and screening participation[87]. A cost-utility analysis comparing SEPT9, FOBT, FIT, sigmoidoscopy, and colonoscopy suggested that while the use of SEPT9 appeared cost-effective for screening compared to no screening, to be cost-effective relative to other established methods SEPT9 or any other blood-based biomarker with similar test performance characteristics would need to achieve substantially higher uptake and adherence rates than the alternatives[88].

Moreover, SEPT9 blood testing raises concerns for potential abuse leading to inadequate screening, a similar drawback is seen with the prostate specific antigen for prostate cancer. Lastly, much like the other non-invasive tests a second intervention is needed if the test was positive[89].

CRC SCREENING PROGRAM ATTRIBUTES

Stratifying the population by colon cancer risk offers the potential to improve the efficiency of screening and helps establishing a screening program. A quick review of the literature reveals more than 50 proposed risk scores for colon cancer that have the potential to identify individuals at high risk. A recent systematic review that examined all the available risk scores, showed that the discrimination of the models, compare favorably with risk models used for other cancers, and several include only variables recorded in routine medical records. Grouping risk models according to type and number of variables included also showed that there is no clear improvement in discrimination as increasing numbers of variables are added from self-completed questionnaires to routine data. A small number of risk models developed from case-control studies of genetic biomarkers showed serious promise but still require further external validation in population-based samples. This review also showed that risk models exist, with the potential to stratify the general population into risk categories, and allow screening and preventive strategies to be targeted at those most likely to benefit, while leaving those at low risk of disease unexposed to direct and indirect side effects of screening programs. This might improve the

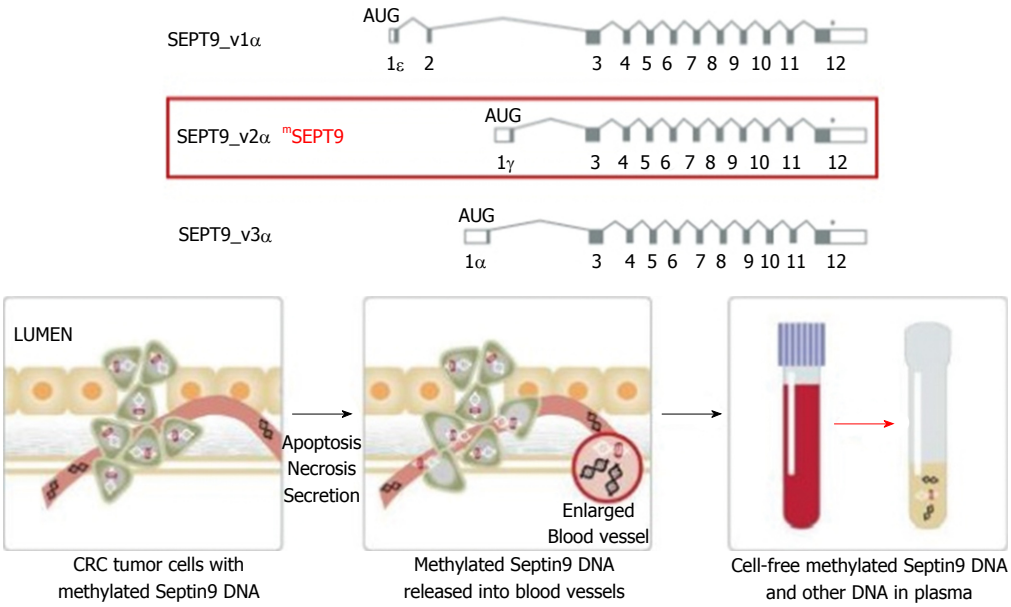

Figure 1 ***SEPT9* gene.** Septins: Multifunctional "scaffolding" proteins that provide structural support during cytokinesis. *SEPT9* gene produces septin-9 that appears to act as a tumor suppressor which is active in cells throughout body. In CRC cells: *SEPT9* gene is hypermethylated and the DNA is released into peripheral blood. Methylated SEPT9 DNA can be detected by PCR 34.

cost-effectiveness of CRC screening and would address concerns about demand and capacity for colonoscopy. The use of risk prediction models would also potentially increase acceptance of screening and provide an opportunity to give information to encourage lifestyle modification[90]. The two most commonly used and validated scores are the Cleveland Clinic test and the NCI test; which are both self-completed questionnaire and can provide a suggested 10-year risk assessment. A prospective examination of the relationship between predicted 10-year CRC risk and the prevalence of advanced neoplasia (AN), defined as advanced or multiple (⩾ 3 adenomatous, ⩾ 5 serrated) adenomas or sessile serrated polyps, in individuals undergoing screening colonoscopy was studied in 509 screeners. AN was found in 11%. The prevalence of AN increased progressively from 6% in the lowest risk-score quintile to 17% in the highest risk-score quintile (P = 0.002). The discriminatory accuracy of the tool was modest, with AUC of 0.61 overall (95%CI: 0.54-0.69)[91].

Despite the various modalities offered for CRC screening, it is still underused by populations. Screening rates have not increased appreciably since 2010 and remain at approximately 60%[92]. Several complex factors play a role in affecting the patients participation and sustained adherence. Barriers to screening include elevated cost, lack of proper education regarding CRC, under appreciation of the benefit of screening, a sense of fatalism, or simply fear of the screening tests[93,94]. Several interventions used in randomized, controlled trials have been shown to increase patient participation rates; such interventions include sending patients invitations from their primary care provider, sending reminder letters, making telephone calls, and mailing fecal occult blood test kits to patients' homes.

Ladabaum *et al*[88] suggested through an elaborate markov model reproduction that at comparable screening participation rates among different strategies, FIT or screening colonoscopy are likely to be more effective and less costly than MT-sDNA. They concluded that in comparison to an organized screening program of yearly FIT, a program of MT-sDNA testing every 3 years could be cost-effective if this program could accomplish a steady participation rate of approximately 66%[82]. Several National screening programs that examined patterns of CRC screening using gFOBT and FIT concluded that there is 20%-29% rate of non-responders and after 3 or 4 cycles there is an additional drop out of up to 30%[95-98]. For instance in an annual FIT screening program in California there was a 48% initial participation with a 75% adherence after 4 cycles[99]. Analysis from those programs also indicate a strong sex and socioeconomic inequalities in CRC screening uptake. Repeated invitations to screening successfully engage previous non-responders. Many respond to at least one screening invitation over multiple rounds with a considerably smaller number of persons responding consistently to all invitations. Therefore, efforts to increase (continued) engagement among these "at-risk" groups are essential to optimize the long-term benefit of organized screening programs[95].

Total patient support, which can include navigation, has emerged as an important component of many CRC screening efforts. The most successful programs use patient navigators to reduce logistic barriers, address cultural issues, and encourage participants to undergo screening. Moreover, patient support costs in the CDC and Prevention's Colorectal Cancer Screening Demonstration Program averaged $153 per person over approximately 1 year[100]. The use of patient

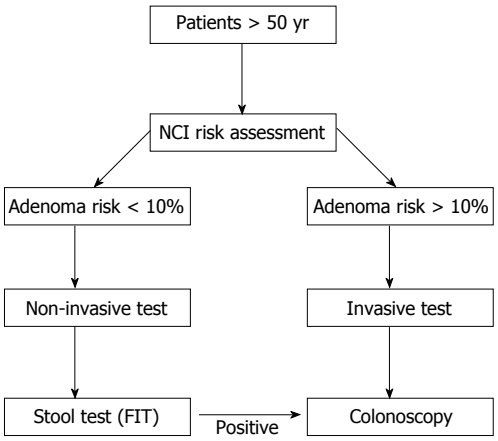

Figure 2 Proposed algorithm for a cost-effective screening program. FIT: Fecal immunochemical test.

navigators is especially important in underserved populations[101,102]. It is estimated to be likely cost-effective and increase people's adherence[103-106].

The National Colorectal Cancer Round table has established a goal of 80% adherence to CRC screening programs by the year 2018. Kaiser Permanente has implemented a comprehensive strategy focused on FIT screening, with colonoscopy performed as follow-up to a positive test. They have reached and maintained the goal of 80% adherence rate through four rounds of screening[99]. Adherence to screening tests varies among strategies, and preference of strategy varies by race and ethnic group; data have shown that white participants more commonly prefer colonoscopy, and non-white participants tend to prefer fecal testing[93,107]. To achieve the highest level of adherence to CRC screening, it may be best to provide participants a choice, because the "best" strategy is the one that they will adhere to consistently.

Maximizing the benefit of CRC screening requires a programmatic approach to implementing screening strategies. The quality of a screening program should be measured by its ability to identify patients who are due for screening, provide access to screening, assess adherence to the screening test and to a follow-up colonoscopy if a non-invasive screening test is positive. It also needs to document test outcomes and disseminate accurate follow-up recommendations, identify patients with a negative test to follow them up for repeat screening at the appropriate intervals, and provide timely surgery for cancers. The rate of adenoma detection (the percentage of patients in whom precancerous polyps are detected during screening colonoscopy) differs substantially among endoscopists and may be used as a measure of the ability of screening to prevent CRC[108]. A retrospective study showed that for every 1% increase in the rate of adenoma detection, there is a 3% decrease in the rate of cancer developing after a colonoscopy[109]. One important factor to increase the adenoma detection rate is the adequate bowel cleansing. Therefore, bowel preparation is considered a crucial step in any CRC screening program. Patient compliance with various bowel preparation procedures remains one of the most difficult tasks to achieve before a colonoscopy. Failure to complete the preparation has several drawbacks in terms of decreased cecal intubation rates, increased rates of missing important lesions, and prolonged colonoscopy duration further increasing patient discomfort[27-30]. Tepeš *et al*[110] evaluated the effectiveness of bowel cleansing with magnesium sulphate and low-volume polyethylene glycol with electrolytes in addition to the effect of timing of the colonoscopy. They found excellent bowel cleansing in 82.61% participants and in more participants with young age with colonoscopy performed in the afternoon[110].

One of the National CRC screening programs was conducted in Slovenia with promising results. They used the FIT test with a positive result being followed by colonoscopy. Assessment of the first round showed an adherence rate of 56.9%. The overall adenoma detection rate was 51.3%. CRC was found in 6.2% of participants who underwent colonoscopy. A localized clinical stage was found in 70.2% of cases. Cancer was cured in 22.8% of CRC patients with endoscopic resection only[111].

Figure 2 illustrates a proposed cost-effective algorithm for a screening program for low-resources countries using the NCT CRC screening risk score.

CONCLUSION

A multitude of options currently exist for CRC screening. A quick review reveals wide variability between programs all over the world. Additionally, one should not forget that most screening, especially in low income countries, is still performed on opportunistic basis with no solid structure. CRC screening must be optimized to reach the golden target of reducing incidence of the disease and eventually mortality. Most importantly we need to achieve high rates of participation and adherence in different screening programs by seeking correction of all the confounding factors. Benefiting from all the available screening tools in the correct settings of each population will increase the compliance of different populations. Consistent with this goal, adoption of cost-effective non-invasive methodologies designed to reduce complications, reduce anxiety over CRC screening, and improve overall acceptance of the screening process would be highly desirable.

Despite the current limitations and caveats, blood based markers may have a solid future. Screening with a relatively inexpensive serum or plasma marker (or marker panel) could increase screening compliance and be cost-saving if participation and performance were both elevated. Such a marker has the potential to replace more cumbersome stool based test in programs that employ a 2-stage paradigm. All the initial enthusiasm raised by the new blood marker

have now faded as the rather disappointing results from the prospective trials were published. At this point due to the significant variability in sensitivity and specificity between various trials, it is very difficult to recommend it for mass screening. Other potential options include the noninvasive stool tests and in that regard the FIT seems to still be the most appropriate due to the present prohibitive cost of the multi-strain stool DNA test and the lack of significant difference in their performance. Colonoscopy is still the most appropriate test in high risk individuals or as second procedure following a positive first test.

Future studies should not only focus on the statistical performance of different tests but also on the characterization of complete screening programs, from the invitation to screening to the completion of colonoscopy for patients with a positive test.

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REVIEW

Therapeutic potential of flavonoids in inflammatory bowel disease: A comprehensive review

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Abstract

The inflammatory process plays a central role in the development and progression of numerous pathological situations, such as inflammatory bowel disease (IBD), autoimmune and neurodegenerative diseases, metabolic syndrome, and cardiovascular disorders. IBDs involve inflammation of the gastrointestinal area and mainly comprise Crohn's disease (CD) and ulcerative colitis (UC). Both pathological situations usually involve recurring or bloody diarrhea, pain, fatigue and weight loss. There is at present no pharmacological cure for CD or UC. However, surgery may be curative for UC patients. The prescribed treatment aims to ameliorate the symptoms and prevent and/or delay new painful episodes. Flavonoid compounds are a large family of hydroxylated polyphenolic molecules abundant in plants, including vegetables and fruits which are the

major dietary sources of these compounds for humans, together with wine and tea. Flavonoids are becoming very popular because they have many health-promoting and disease-preventive effects. Most interest has been directed towards the antioxidant activity of flavonoids, evidencing a remarkable free-radical scavenging capacity. However, accumulating evidence suggests that flavonoids have many other biological properties, including anti-inflammatory, antiviral, anticancer, and neuroprotective activities through different mechanisms of action. The present review analyzes the available data about the different types of flavonoids and their potential effectiveness as adjuvant therapy of IBDs.

Key words: Antioxidant; Inflammation; Gastrointestinal tract; Flavonoids; Polyphenols

Core tip: Inflammatory bowel diseases (IBDs) involve inflammation of the gastrointestinal tract and primarily comprise Crohn's disease and ulcerative colitis. Currently, there is no cure for most of the IBDs. Emerging evidence suggests that flavonoids have many biological and pharmacological properties, including anti-inflammatory, antiviral, anticancer, and neuroprotective activities through different mechanisms of action. The present review critically analyzes the current experimental evidence on the therapeutic potential of flavonoids in IBD.

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INTRODUCTION

Inflammation is a protective and complex process consisting of a set of molecular, cellular and vascular defensive responses against any injury, including chemical, physical, or biological attacks, and focused on restoring tissue function[1]. Inflammatory diseases comprise a group of illnesses characterized by a long-term pro-inflammatory state[2]. A large number of pathologies are considered as inflammatory diseases, such as autoimmune and cardiovascular disorders, chronic obstructive pulmonary diseases, neurodegenerative diseases, and chronic inflammatory bowel disease (IBD). This inflammatory response is associated with changes in vascular permeability, increases in blood flow, leukocyte mobilization and rise in the production of inflammatory mediators[3,4]. Some of the produced mediators, mainly cytokines, are able to activate signaling by nuclear factor-kappa B (NF-κB), a transcription factor which also mediates the inflammatory response[5-7].

IBDs include a group of pathologies characterized by chronic and uncontrolled inflammation associated with deregulation of both adaptive and innate immunity that affects the gastrointestinal tract[8,9]. The bowel inflammation results in symptoms, such as abdominal pain, bleeding, recurrent diarrhea and weight loss[8,9]. The two main pathologies are Crohn's disease (CD) and ulcerative colitis (UC). CD can affect any area of the gastrointestinal tract, from the mouth to the anus, although the ileum is the most affected section. In contrast, UC primarily affects the colon and the rectum. The exact cause of IBD is not fully known, although there is an interaction between diverse factors, such as an immune system disturbance, genetic predisposition, and environmental factors, which activates the damaging immune response in the intestines. Today, there is no effective pharmacological treatment that allows for cure of the disease. Medical therapy is focused on non-specific immunosuppressive therapies, including thiopurines and methotrexate[10,11]. The occurrence and prevalence of IBDs are progressively growing in all areas around the world, suggesting its appearance as a global disease in the near future[12].

The term flavonoid derives from the Latin word "flavus", meaning yellow, and comprises a group of secondary metabolic compounds widely found in plants well known for the distinctive blue, red, and purple anthocyanin pigments of their different structures. Although they are not stated as nutrients and regardless of their physiological functions in plants, flavonoids are key ingredients of the human diet[13]. Based on epidemiological studies, diets rich in flavonoids are in direct correlation with increased longevity and decreased cardiovascular disease incidence, despite consuming diets with high fat content[14-17]. Many biological effects have been attributed to the flavonoids, in addition to their antioxidant properties, some of which include anti-inflammatory, antimicrobial, vasodilatory, anti-ischemia and anticancer effects[16,18-20].

Recently, owing to their significant antioxidant and free radical scavenging properties observed *in vitro*, interest towards investigating new possible health benefits has significantly increased. In fact, flavonoids are of great nutritional value in inflammatory diseases because they can block many pro-inflammatory proteins and can be considered natural inhibitors of inflammation, ameliorating the intensity of inflammation[21]. In addition to the direct antioxidant activity, flavonoids are capable of activating diverse antioxidant and protective genes *via* nuclear transcription factors and also of inhibiting inflammatory pathways[22]. Flavonoids influence the composition of the microbial flora, favoring the growth

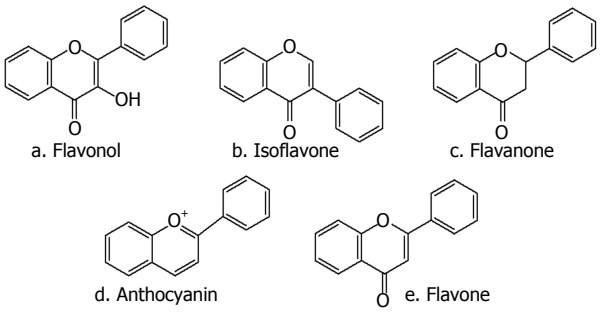

Figure 1 Chemical structures of the backbones of flavonoids.

of bifidum and lactobacilli bacteria and stimulating an anti-inflammatory environment[23-26].

As described earlier, since the precise etiology of IBD has not been identified clearly yet and no specific causal treatment has been established thus far, based on the aforementioned biological effects, flavonoids may be of great utility in managing IBD. It should be noted that another review paper broadly covered this topic during the past year[27]. In this review, we discuss the different subclasses of flavonoids and existing data about their effectiveness in preclinical models of IBD, which could translate to a future role in human IBD therapy.

FLAVONOLS

Flavonols are one of the main subclasses of flavonoids, with the specific 3-hydroxyflavone structural backbone depicted in Figure 1A. Multiple flavonols have been extracted from leaves, flowers and the outer part of plants and their pharmacological effects have been evaluated through several studies. The most well-known members of this group of flavonoids are quercetin (3,3′,4′,5,7-pentahydroxyflavone), rutin (quercetin 3-rutinoside), morin and kaempferol (Figure 2)[28].

Quercetin, quercitrin and rutin

Quercetin and its glycosylated derivatives, such as rutin and quercitrin (quercetin 3-rhamnoside), are the foremost representatives of flavonols, demonstrating remarkable effects on attenuation of pharmacological models of colitis[29-32]. Although many *in vitro* studies have determined that quercetin is more effective than its glycosylated derivatives in reducing the inflammatory response, the majority of *in vivo* studies did not observe this same efficacy. In this way, it was reported that a diet with 0.1% rutin in its composition supplied during 2 wk, but not quecetin, ameliorated dextran sulfate sodium (DSS)-induced colitis in a mouse model *via* lessening of pro-inflammatory cytokine production[29].

Poor stomach[33] and intestinal absorption[34,35] of these compounds are the primary obstacles against reaching an adequate concentration in colon. Many studies investigating the gastrointestinal absorption of flavonols have suggested that the hydrophilic structure of quercitrin and rutin is the main cause of their poor absorption. Present in colon, glycosylated flavonols are cleaved by colon microflora forming the aglycon shape of these compounds[36-38]. Therefore, it is suggested that quercitrin and rutin can act as pro-drugs of quercetin, preserving the aglycon moiety from absorption and assuring an intact nature in the colon and ability to reach where it will be further hydrolyzed and yield quercetin[34,39,40]. It seems that colon-specific drug delivery systems are a necessary strategy to preserve quercetin from degradation and absorption through the gastrointestinal tract and to increase its availability. Castangia *et al*[41] demonstrated that chitosan/nutriose-coated vesicles represent a promising strategy to improve quercetin concentration in the colon. Additionally, Guazelli *et al*[30] depicted that quercetin-encapsulated microcapsules are more effective in pharmacological animal models of colitis compared to intact quercetin.

In a study performed on acetic acid-induced colitis in mice, treatment with quercetin (100 mg/kg) loaded pectin/casein polymer microcapsules significantly prevented the depletion of glutathione (GSH) reservoirs in the colon. Although the results did not evidence significant statistical differences between treatment and control groups, at least a significant tendency for preserving GSH reservoirs was observed[30]. According to Dodda *et al*[42,43] administration of quercetin (50 and 100 mg/kg, intra-rectal) in tri-nitrobenzene sulfonic acid (TNBS)- and (50 and 100 mg/kg, p.o.) in acetic acid-induced colitis rat models resulted in a considerable elevation in the GSH levels when compared with the control group. A main limitation of these two studies is the use of high concentrations of quercetin that cannot be achieved with a normal diet. With regard to quercitrin, the oral administration of 1 and 5 mg/kg at 2 h before TNBS-induced colitis in rats thwarted GSH depletion[44]. Also, in two separate studies, Sánchez de Medina *et al*[44] and Cruz *et al*[45] showed that oral pretreatment of rats with 1 and 5 mg/kg quercitrin and 5, 10 and 25 mg/kg of rutin increased GSH levels in both acute and chronic phase of TNBS-induced colitis.

Other mechanisms for ameliorating flavonols effects on pharmacological models of colitis include the suppression of nitric oxide (NO) production and/or inducible nitric oxide synthase (iNOS) expression. Camuesco *et al*[46] proposed that the histological and biochemical anti-inflammatory effects of quercitrin might be related to a decrease in iNOS expression through down-regulation of NF-κB in colonic tissue. They also demonstrated that oral administration of quercitrin (1 and 5 mg/kg) significantly reduced the iNOS expression, contributing to the inhibition of iNOS activity. This outcome had been supported by subsequent studies.

A down-regulation of the inflammatory response of macrophages derived from bone marrow, inhibition of

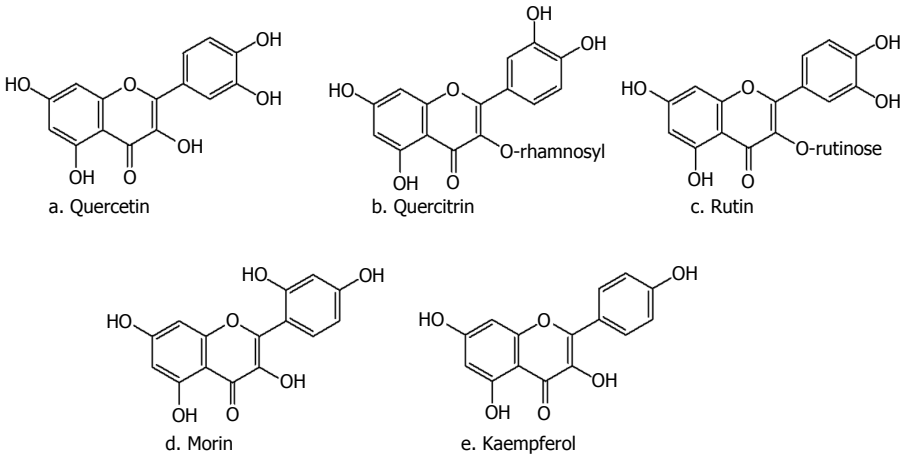

Figure 2 Chemical structures of the various flavonols.

cytokine and NO synthase expression through inhibiting the NF-κB pathway in the presence of quercetin and quercitrin (1 mg/kg/d, 15 d) was reported in an experimental rat model of colitis provoked by DSS. This study also suggested that the effects of quercitrin observed *in vivo* might originate from the release of quercetin by intestinal microflora[31]. In two distinct studies, the *in vitro* inhibitory effects of quercetin on NO production were demonstrated in lipopolysaccharide (LPS)-induced macrophages. In both studies, the expression of mRNA and protein of NOS was attenuated in cell cultures and this effect was attributed to suppression of the NF-κB pathway[47,48].

In another study, a diet containing 0.1% rutin mitigated the DSS-induced weight loss and improved colitis histological scores in mice probably through inhibition of interleukin (IL)-1β and subsequent inhibition of the induction of iNOS in enterocytes[29]. Also, oral administration to rats with TNBS-induced colitis of rutin (10 mg/kg) for 6 d had significant ameliorating effects on inflammation of the colon, with similar effectiveness as sulfasalazine (30 mg/kg), and also decreased myeloperoxidase (MPO) activity[40]. Finally, rutin (28.5 and 57 mg/kg per day by gavage) also demonstrated anticolitis activity, when examined in a mouse T-cell transfer model of IBD[49].

The main limitation of quercitrin and rutin in IBD is the fact that the compounds represent only a small fraction of the flavonoids usually ingested in the diet, being probably insufficient to exert a significant pharmacological effect. For that reason, the development of pharmacological formulations containing concentrations that can be quantitatively active and therapeutic is required.

Morin

As a flavonols family member, morin is present in a variety of fruits, vegetables and beverages[50]. In several studies, antioxidant, anti-inflammatory, anticancer, antidiabetic, and cytoprotective effects of this compound were assessed[51]. The effects of morin were evaluated in acute and chronic stages of the TNBS-induced colitis. In the acute model of colitis, pretreatment with morin (25 mg/kg) significantly alleviated the intestinal inflammation *via* inhibition of colonic leukotriene B4 (LTB4) production and due to its antioxidant properties[52]. In the chronic phase of colitis, the administration of morin (25 mg/kg) exhibited significant anti-inflammatory effect *via* attenuating production of inflammatory mediators, such as free radicals, LTB4, NO and IL-1β[53]. Although the authors confirmed that morin showed inhibitory effects against colonic NO synthase activity in an *in vitro* assay, the specific pathway mediating the anti-inflammatory effects has been not investigated. Finally, the authors stated, similar to quercitrin and rutin, that the amounts of morin employed in this study were notably higher than that attained through dietary consumption.

Kaempferol

Kaempferol is abundant in plants of the genera *Delphinium*, *Camellia*, *Berberis*, *Citrus*, *Brassica*, *Allium*, *Malus*, *etc.*[54], and similar to other flavonoids is naturally bond to different sugars[55]. This flavonol possesses different biological activities, such as anticancer[56-61], antimicrobial[62,63], antioxidant[64-68], and anti-inflammatory[69-71]. According to Calderón-Montaño *et al*[55], kaempferol's anti-inflammatory property is mostly derived from its ability to inhibit NF-κB, activator of transcription 1 and activator protein 1 pathways that regulate a wide spectrum of genes, including cytokines, growth factors, stress-response proteins[55,71]. Inhibition of these pathways is associated with a decrease in tumor necrosis factor-alpha (TNF-α) levels, IL-1β and IL-8 expression, cyclooxygenase-2 (COX-2), lipoxygenase and iNOS activation and with a reduction of cellular levels of reactive oxygen species[55]. Also, Park *et al*[72] reported that 0.3% kaempferol administered pre- and post-feeding diminished DSS-induced colitis

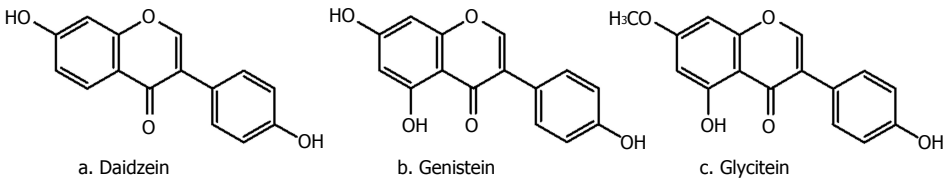

Figure 3 Chemical structures of several isoflavones.

in mice through down-regulation of TNF-α, IL-6, IL-1β, NOS, and COX-2 at the mRNA expression level. In addition, the compound reduced LTB4, prostaglandin E_2 (PGE_2) and NO levels and MPO activity. Furthermore, in the prefer group, kaempferol preserved the goblet cell function, which was indicated *via* up-regulation of trefoil factor family 2 mRNA expression in the distal colon mucosa[72].

ISOFLAVONES

One of the main subclasses of flavonoids, mostly found in soybeans, nuts and whole grains are isoflavones. These naturally occurring compounds are glycoside conjugates, predominantly malonyl-glycosides, and have a common backbone of 3-phenylchromen-4-one (Figure 1B)[73]. During different stages of food processing, including fermentation or hot water extraction, the glycosidic groups of genistin, diadzein and glycitinis (Figure 3) are removed[74]. After ingestion, intestinal enzymes and/or intestinal microflora hydrolyze the conjugated isoflavones and produce more bioactive and bioavailable genistein, diadzein and glycitein[74,75]. Afterwards, these unconjugated aglycones are either passively absorbed through the small intestine or metabolized to other metabolites, such as equol, P-ethyl phenol and dihydroglycitein, consistent with diadzein, genistein and glycitein, respectively[76,77].

Along with antimicrobial, antioxidant, anti-inflammatory and anticancer activities, isoflavones can also reduce the risk of cardiovascular diseases and osteoporosis[75]. Additionally, the structural similarity of these compounds with estrogens, specifically 17-β estradiol, allows them to act as partial estrogen receptors (ERs)[77,78].

Some studies have demonstrated that estrogen receptors, especially ER subtype beta (ER-β), play a key role in improving the epithelial intestinal barrier[79,80]. Moussa *et al*[80] reported that daily treatment with 0.45 mg of fermented soy germ ingredient (FSG), mainly consisting of isoflavones and Bowman-Birk inhibitors (BBI, a serine protease inhibitor), significantly suppressed TNBS-induced colitis in rats *via* two different mechanisms. First through ER-signaling of isoflavones, which is able to reduce inflammatory cytokines, and second through protease-activated receptor - mediated pathway ascribed to BBI. In a recent study, FSG suppressed macrophage migration inhibitory factor production which in turn down-regulated the IL-1β. In addition, FSG can elevate the level of IL-10 and limit gut permeability in colitis. The FSG effects have been antagonized by administration of ER antagonist, proposing that the FSG effects are mainly mediated by ER-β[80].

Activation of the ER pathway is associated with an increased expression of membrane tight junction proteins, which can improve the intestinal barrier integrity, and decreased pro-inflammatory cytokine release. This anti-inflammatory response can be mediated by increased release on the anti-inflammatory IL-10, which in turn inhibits pro-inflammatory cytokine release. Finally, it is interesting to note that these evidenced effects took place when using doses of isoflavones near to the daily ingestion authorized in humans (1 mg/kg body weight/d).

Similar results were obtained using fermented *Peuraria lobata* extract, rich in isoflavones, in ameliorating the gastrointestinal barrier function in colitis induced by DSS. A reduction of inflammatory cytokines' mRNA expression and recovery of construction and expression of tight junction proteins was observed *in vitro*. Additionally, restoration of goblet cells and improvement of epithelial structure in the colonic mucosa was reported *in vivo*[81]. Also, the isoflavone rich fraction of soybean extract inhibited IL-8 production through TNF-α suppression in the Caco-2 cell line in a dose-dependent manner[82]. In this way, IL-8 release induced by hydrogen peroxide or by IL-1β was not ameliorated when cells were treated with the extract, indicating the implication of TNF-α.

As mentioned above, genistein is another well-characterized aglycon isoflavone. The efficacy of this compound in experimental IBD models has been studied in various experiments. Similar to other isoflavones, genistein can bind to ERs, especially ER-β, the most abundantly expressed subtype in the gastrointestinal tract mimicking estrogenic action[83]. Various studies have demonstrated that ER ligands possess the ability to attenuate IBD symptoms[84,85]. Inflammation-mediated mechanisms have been widely described for protective effects of genistein.

In the study performed by Seibel *et al*[86] it was demonstrated that genistein oral administration (100 mg/kg body weight) in TNBS-induced colitis in rats resulted in a significant reduction in MPO activity and COX-2 mRNA expression[83]. The authors hypothesized that the treatment with genistein can inhibit the

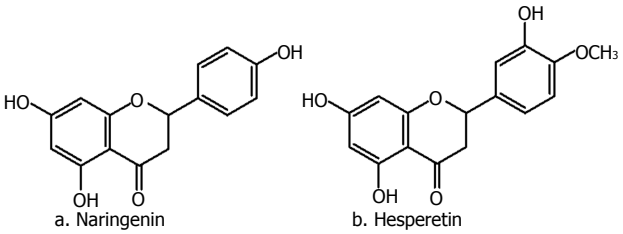

Figure 4 Chemical structures of different flavanones.

expression of MPO and COX-2 by means of an ER-dependent mechanism, inhibiting the activation of the NF-κβ signaling pathway. Contrarily, Seibel *et al*[86] informed that in uterus and postnatal rats, exposure to a phytoestrogen-rich diet (including high and low dose of genistein and daidzein) not only did not protect the offspring from TNBS-induced colitis but also enhanced the extent of acute inflammation through increasing neutrophil infiltration and COX-2 protein expression. These surprising results may be attributed to the fact that COX-2 expression is partly repressed by phytoestrogens in the acute phase of inflammation, exacerbating colon inflammation. The authors suggest as a possibility that most of the positive results were obtained in chronic models of IBD and, consequently, it may be an initial magnification of the inflammatory response followed by an enhanced anti-inflammatory process.

Furthermore, based on another *in vitro* study, genistein significantly prevented the xanthine oxidase/xanthine-induced oxidative stress-mediated alteration in paracellular junctional protein complexes, such as tyrosine phosphorylation and their disassembly from the junctional complex, thereby protecting tight junctions from malfunction in Caco-2 cells[87]. Also, the administration of genistein significantly inhibited the disruption of tight junction by acetaldehyde through deterring tyrosine phosphorylation in Caco-2 cells[88]. The mechanism of action is related to genistein activity as tyrosine kinase inhibitor, which is capable of reducing the tyrosine phosphorylation of functional proteins and also of protecting against dissociation of these proteins from the cytoskeleton[87,88]. Moreover, these protective effects of genistein have also been observed against intestinal tight junction barrier damage triggered by inflammatory mediators, including TNF-α and enteric bacteria, such as *Escherichia coli, Proteus mirabilis, Listeria monocytogenes* or *Salmonella typhimurium*[89,90].

Sergent *et al*[91] revealed an inhibitory effect of genistein on IL-6 and monocyte chemoattractant protein-1 (MCP-1) overproduction in a model of inflamed human intestinal epithelium when investigating the anti-inflammatory activity of some phenolic molecules. In the same study, genistein also down-regulated the levels of NOS and 14 different inflammatory genes[91]. In another study, genistein supplied in dietary concentration thwarted the overproduction of TNF-α and IL-6 in RAW 264.7 macrophages treated with LPS[92]. The ameliorated inflammatory response seems to be mediated by inhibition of NF-κβ activation following AMPK phosphorylation.

Diadzein is another important bioavailable isoflavone exhibiting protective effects in mice with DSS-induced colitis *via* suppressing the expression of IL-6, IL-8, IL-12, p40 and interferon-gamma (INF-γ) and triggering IL-10 secretion from mesenteric lymph node cells[93]. The authors also reported that diadzein inhibited cytokine production in human monocytic cell lines after Toll-like receptor (TLR)-2 and TLR-4 stimulation, suggesting that the TLR signaling pathway could be a target for isoflavones effects[93]. However, in contrast, equol, the metabolite of diadzein, perpetuates colitis in the DSS-induced model *via* an unknown mechanism[94]. The treatment timing could be responsible for the negative results since isoflavones can potentiate inflammation in acute colitis, as was mentioned above. In this study, biochemical analysis was performed on day 5 after initiating the colitis treatment, whereas most of the studies reporting beneficial effects lasted more than 14 d since the colitis induction. This possibility is reinforced by the fact that genistein did not improve the severity of colitis[94].

FLAVANONES

Hesperidin and naringin (Figure 4), like other flavonoids found naturally as glycosides[95], are common constituents of citrus fruits[96]; their skeleton is represented in Figure 1C. Naringin (naringenin-7-*O*-neohesperidoside) and hesperidin (hesperetin-7-rutinoside) are mainly hydrolyzed by the microflora of the distal part of the small intestine and colon into their aglycones, naringenin and hesperetin, respectively[97].

Various *in vivo* and *in vitro* investigations have evidenced the therapeutic activity of naringin, naringenin, hesperidin and hesperetin in several pathological conditions, including cancer, cardiovascular and neurological disorders, and diabetes mellitus[96,98,99] that might be attributed to the anti-inflammatory and antioxidant effects of these constituents.

Kumar *et al*[100] evaluated the efficacy of naringin in acetic acid-induced colitis models and reported that administration of naringin in different concentrations significantly attenuated inflammatory responses, which in turn prevented further DNA damage. This study also demonstrated that the malondialdehyde (MDA), MPO, NO, xanthine oxidase and alkaline phosphatase concentrations were significantly decreased after naringin treatment, with respect to the control group. Furthermore, this study also reported a reduction of ulcer lesions by naringin, suggesting a potential protection of colonic microflora from the corrosive effect of acetic acid.

Other studies have also demonstrated that

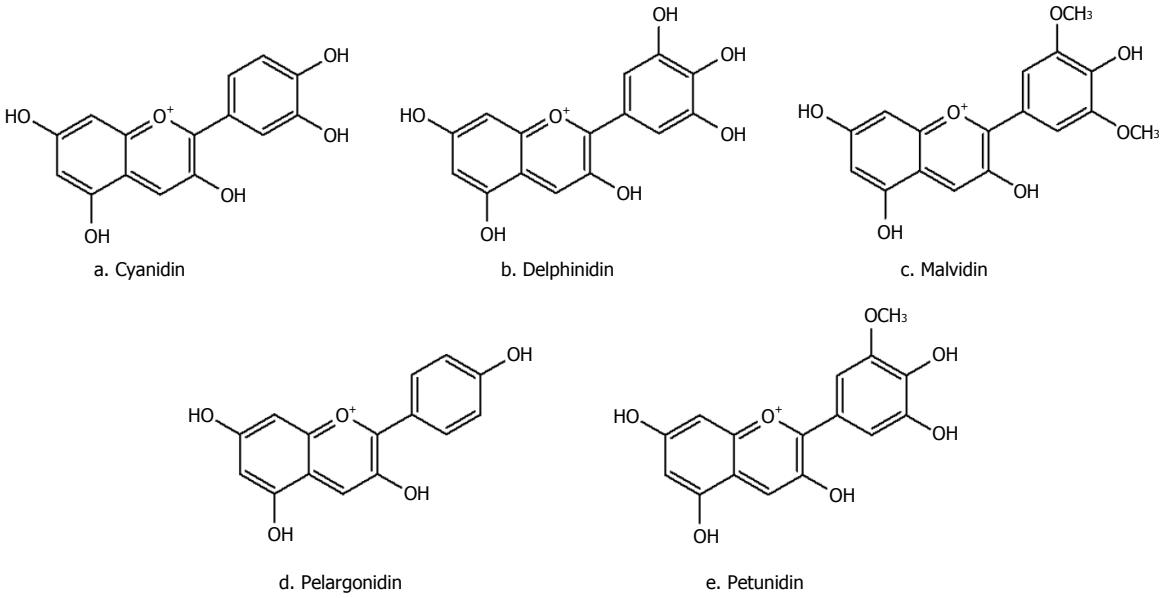

Figure 5 Chemical structures of several anthocyanins.

naringenin can ameliorate colitis in different animal models of colitis. In a remarkable study conducted by Dou *et al*[101], it was demonstrated that naringenin treatment significantly improved colitis through down-regulating the mRNA expression of several proinflammatory mediators, including iNOS, MCP-1, intercellular adhesion molecule-1, COX-2, IL-6 and TNF-α. Additionally, they found that the administration of naringenin significantly inhibited up-regulation of TLR-4 expression, which in turn reduced pro-inflammatory cytokines, especially IL-6 and TNF-α. Moreover, phospho-NF-κB p65 protein levels were also decreased, correlating with decrease in phospho-IκBα protein concentrations. These data were consistent with *in vitro* results obtained by the same investigators[101].

Still other studies have demonstrated the effects of naringenin in different concentrations on colitis models *via* inhibition of INF-γ, macrophage inflammatory protein 2 (MIP-2), PGE_2, NO, IL-6, IL-17A and IL-1β expression[102,103]. Furthermore, naringenin can protect the tight junction barrier[102]. Altogether, these results seem to indicate that targeting of the TLR-4/NF-κB signaling pathway might be one of the underlying mechanisms implicated in the protective effects of naringenin against IBD.

Xu *et al*[104] demonstrated that hesperidin, just like naringenin, exhibited its beneficiary effects in DSS-induced colitis *via* decreasing MDA contents, MPO activity, and IL-6 expression levels. In addition, hesperidin reduced iNOS activity and production of PGE_2 and NO in a mouse macrophage cell line[104]. However, based on the present data, the explanation of the possible mechanism by which hesperidin acts is difficult to clarify.

ANTHOCYANINS

As the red pigment of different berries, currants and grapes, anthocyanins are composed of an anthocyanidin [malvidin, cyanidin, pelargonidin, petunidin, delphinidin, *etc.* (Figure 5)] conjugated with one to three sugar molecules, including glucose, galactose, xylose, arabinose and rhamnose[105]. In most cases, anthocyanins absorption is dependent on the structure of their aglycone moiety. This group of natural compounds is not regularly metabolized; however, in acidic media, they become rearranged and more stable[106]. Overall, anthocyanins are only partially absorbed and have demonstrated limited biological activity on enterocytes.

Various investigations have been centered on the antioxidant activity of anthocyanins; however, the anti-inflammatory effect has also been extensively observed in other non-intestinal tissues[107-109]. Based on a randomized trial with human participants on a dietary regimen rich in purple-flesh potatoes, containing high amounts of anthocyanins, a significant rise in serum antioxidant parameters levels and a decrease in pro-inflammatory markers, such as IL-6 and C-reactive protein, was reported[110]. Other studies have also reported radical scavenging and anti-inflammatory activities of anthocyanin-containing natural compounds, including bilberry juice, press cake and soybean seed[111,112].

The main limitation in studies testing anthocyanins is the fact that fruit extracts rich in these compounds were used, but not the isolated compounds themselves. All these extracts are an excellent source of vitamin C and other antioxidant compounds, in addition to anthocyanins such as non-flavonoid condensed tannins[113]. As the composition of these extracts was

not carried out in those studies, it cannot be specifically concluded that anthocyanins are solely responsible for the protective effects against IBD.

Strawberry anthocyanins

Strawberry as a member of the Rosaceae family, and is a fruit rich in anthocyanin compounds derived from pelargonidin and cyanidin aglycones[114,115]. The anti-IBD effects of strawberry anthocyanins have been mostly attributed to the free-radical scavenging and anti-inflammatory properties of these compounds[116,117]. Based on an *in vivo* study on acetic acid-induced colitis in rat, oral or rectal administration of strawberry significantly decreased the infiltration of polymorphonuclear cells to the inflammatory site and lessened epithelial necrosis and lesions[118].

Blueberry anthocyanins

As a member of the Ericaceae family, anthocyanins found in blueberry are derivatives of malvidin, delphinidin, cyanidin and petunidin aglycones[119]. Anthocyanins present in blueberry mainly decrease colony number of *Clostridium perfringens* and *Enterococcus* Spp, increase butyric acid concentrations, reduce the amounts of succinic acid, decrease IL-6, TNF-α and IFN-γ, increase IL-10 plasma concentrations, and suppress mucosal congestions and colon wall thickening[120,121].

Cranberry anthocyanins

Cranberry is another member of the Ericaceae family, which is full of anthocyanin compounds. Based on a study performed by Xiao *et al*[122], the administration of cranberry polyphenols was associated with a reduction in colon length, MPO activity, disease progression, infiltration of inflammatory cells and structural damage to the mucosa in an experimental animal model of colitis induced by DSS[122].

Grape anthocyanins

Belonging to the Vitaceae family, grape is rich in anthocyanins, with glycoside forms of malvidin, delphinidin, petunidin, petonidin and pelargonidin[123]. Oral administration of grape juice has been shown to significantly reduce the concentrations of TNF-α and iNOS, and COX-2 enzyme activities. Furthermore, it can reduce peripheral blood genotoxicity and morphological signs of cell damage in colitis[124].

Bilberry anthocyanins

As an Ericaceae family member, among other berries, bilberry fruit possesses the highest amounts of anthocyanins (about 300 to 700 mg/100 g fresh fruit). The main anthocyanins in this fruit include delphinidin, malvidin, cyanidin and petonidin[125]. The anti-IBD mechanisms of this fruit have been proposed to involve suppression of TNF-α, IL-6 and INF-γ secretion, resulting in colon shortening and decreased histological scores, causing intestinal inflammation and ileum mucosal injury[126-128].

Barberry anthocyanins

Barberry is a member of the Berberidaceae family, which is full of anthocyanins, with capabilities of decreasing macroscopic ulcer index and ulcer area, wet weight/length ratio of colon and infiltration of inflammation-inducing cells[129].

Black bean

Phaseolus vulgaris or black bean is another natural dietary food with high levels of anthocyanins which can significantly reduce inflammatory processes in IBD through suppressing the expression of IL-6, IL-9, IL-17a and IFN-γ. Furthermore, these compounds can also decrease IL-1β, IL-17a, TNF-α and IFN-γ serum levels, contributing to a more effective suppression of inflammation in experimental IBD[130].

FLAVONES

Flavones are an important subclass of flavonoids, possessing the backbone of phenylchromen-4-one. The natural compounds present in this subclass, consisting of apigenin, baicalein, luteolin, diosmin, wogonin and tangeretin (Figure 6), are mainly found in foods, medicinal herbs and cereals. Flavones are mainly found as 7-*O*-glycososides; however, C-glycosides (in which the sugar is linked to an aromatic carbon atom) have also been identified. Nevertheless, despite presence of these compounds in teas and cereals, few data exist in regards to the flavone-C-glycosides form of these compounds[131].

Apigenin

Apigenin, present in chamomile, parsley and celery, is the main ingredient of wheatgrass juice. It has been shown to be mostly effective in treatment of UC. This naturally-occurring compound is believed to possess both antioxidative and anti-inflammatory effects, which may be beneficial in the case of IBD therapy. Furthermore, this natural compound has the potency to prevent the transactivation induced by TNF-α[132,133]. Of note, apigenin showed efficacy in a murine DSS colitis model by inhibiting inflammasome pathways and therefore production of IL-1β and down-regulation of iNOS and COX-2, and to reduce serum levels of matrix metalloproteinase-3[134]. However, the underlying mechanism of action was not investigated and additional investigations are required in order to provide the basis for the anti-IBD effects. A randomized controlled trial on managing UC through administration of wheatgrass juice demonstrated that although sigmoidoscopic evaluation did not demonstrate any significant differences among the treatment and control group, other symptomatic indicators of disease activity, such as rectal bleeding, were significantly improved[133].

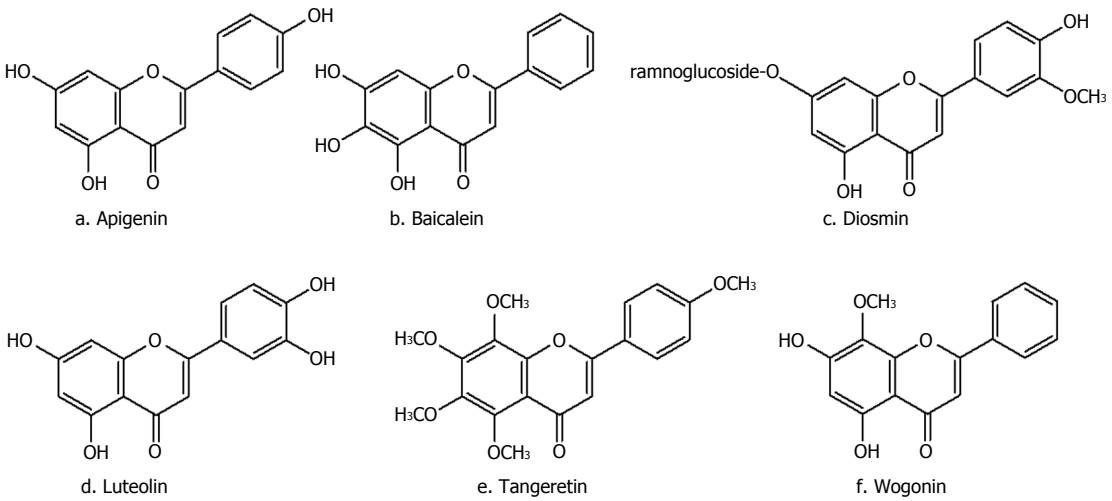

Figure 6 Chemical structures of various flavones.

Baicalein

As the main component of *Suctellaria baicalensis*, baicalein is another member of the flavones group, which is also found in vegetables and fruits. Multiple beneficial activities, including anti-inflammatory, antioxidant and anti-allergic responses, have been reported after administration of this compound in different disorders[135-137]. The plant has also been shown to be significantly effective against experimental IBD. Based on results of a study using a murine model of colitis, investigators found that baicalein could significantly improve colitis inflammatory symptoms, including blood hemoglobin content, rectal bleeding and weight loss, with similar effects to that of sulfasalazine, the reference drug[138]. These effects were also comparable with those observed with wogonin and baicalin[139].

Luteolin

Luteolin, found abundantly in *Salvia tomentosa*, has been shown to significantly lessen shortening of colon length and reduce the histological score of colitis. As reported by Nishitani *et al*[140], luteolin significantly suppressed macrophage and IFN-γ producing $CD4^+$ T cell infiltration into the colon mucosa. Furthermore, the treatment with luteolin significantly improved the mRNA expression of IFN-γ in colon. Additionally, co-culture of intestinal epithelial Caco-2 cells and macrophage RAW264.7 cells submitted to luteolin treatment led to the suppression of *IL-8* gene expression in the intestinal cells without disrupting the epithelial monolayer. Furthermore, TNF-α and pro-inflammatory cytokines' expression, including that of TNF-α, IL-6 and IL-1β, were also significantly reduced in RAW264.7 cells by this compound[140]. The authors proposed that luteolin aglycones are liberated by the Caco-2 epithelial cells inhibiting NF-κβ translocation into the nucleus of RAW264.7 macrophages. This action is followed by suppression of TNF-α gene expression in and release from RAW264.7 cells, leading to reduction in the IL-8 expression in Caco-2 cells.

Diosmin

Diosmin, the main active component found in *Scrophularia nodosa*, was initially isolated in 1925 and applied as therapeutic agent in 1969. Similar to the other members of this subfamily, diosmin has demonstrated several anti-inflammatory, antimutagenic and free radical scavenging effects. Crespo *et al*[141] investigated the anti-inflammatory activity of this compound on the acute phase immune response in a rat model of TNBS colitis and found that pretreatment with diosmin could significantly reduce colonic damage through suppressing MPO enzyme activity, increasing colonic GSH levels and preventing further production of leukotriene B4 and MDA. Additionally, this compound could also inhibit the inflammation and oxidative damage in colitis[141]. The reduction of leukotriene B4 levels is an interesting mechanism of action because this compound is implicated in the pathogenesis of IBD since it strongly promotes neutrophil chemotaxis and activation favoring the inflammatory process.

Wogonin

As an *O*-methylated flavone, wogonin is another flavonoid-like chemical agent found in *Scutellaria baicalensis*[142]. Oroxindin is also the glucuronide form of wogonin isolated from *Oroxylum indicum*[143]. Wogonin has been shown to possess several pharmacological effects, most importantly the anti-inflammatory ones. Based on Wang *et al*[144], Caco-2 cells exposure to wogonin significantly diminished LPS-induced alterations in trans-epithelial electrical resistance and fluorescent markers of transportation. Furthermore, this phytochemical could significantly suppress LPS-induced changes in tight junction proteins, mostly claudin-1 and zonula occludens-1 (ZO-1). Additionally, the expression of IL-6, IL-8, IL-1β,

iNOS and COX-2 was also suppressed by pretreatment with wogonin. The expression of different molecules, including TLR4, MyD88 and TAK1, were significantly suppressed by wogonin. The more interesting finding was that the translocation of NF-κB and its capacity to bind with DNA in LPS-induced Caco-2 cells was also significantly reduced. Consequently, it can be concluded that wogonin, mostly through diminishing the TLR4-mediated inflammatory response and preserving intentional barrier function, could be a potent treatment for IBD[144].

Tangeretin

As the main component of *Citrus* Spp pericarp, tangeretin can effectively inhibit the expression of TNF-α, IL-23 and IL-12 through restricting the activation of NF-κB in LPS-treated dendritic cells. Furthermore, oral administration of this compound was able to suppress the inflammatory process by preventing the activation NF-κB and mitogen-activated protein kinases (MAPK) pathways and lessening MPO activity in mice with TNBS-induced colitis. Tangeretin could also increase the altered TNBS-suppressed expression of several tight junction proteins, such as claudin-1, ocluadin-1 and ZO-1. Additionally, this compound also prevented TNBS-induced type-1 T helper (Th1) and type-17 T helper (Th17) cells' differentiation. Also, tangeretin could inhibit T-bet, RAR-related orphan receptor-γ, IL-12, IL-17 and TNF-α expression. According to the aforementioned results, it can be concluded that oral administration of tangeretin through suppression of IL-12 and TNF-α expression as well as NF-κB activation results in the attenuation of UC[145].

Fisetin

Fisetin is a flavonoid, which can be found in many fruits and vegetables. Recently, this compound showed efficacy against DSS-induced colitis in a mouse model. The mechanism of anti-colitis activity was multifactorial through inhibition of diverse signaling pathways including Akt, p38 MAPK and NF-κB in the murine colon[146]. This study evidenced that fisetin is capable of reducing the LPS-induced phosphorylation of IκBα and NF-κβ (p65) binding activity to DNA. This inhibition was associated with inhibition of upstream proteins related to NF-κβ activation. Specifically, the study reported an attenuation in the phosphorylation of Akt and, therefore, the activation of p38 MAPK and NF-κB in the colon[147].

FLAVANOLS

Flavanols are characterized by the presence of flavan-3-ol as a monomeric unit. Flavanols are usually divided in monomers (or catechins) and condensed tannins (dimers, trimers, oligomers, and polymers)[148]. The group of catechins is composed by flavanols with catechin, epicatechin, gallocatechin, epigallocatechin, and diverse specific gallic acid esters at the 3-OH position. This type of flavonoid is present in notable amounts in a diversity of fruits and beverages, including grapes, lychees, strawberries, cacao and green tea[149]. The bioavailability of flavanols depends on each compound, although absorbed flavanols generally present a short half-life in plasma and undergo an extensive phase Ⅱ metabolism[150,151]. A portion of ingested flavanols is absorbed intact, whereas the remaining fraction is metabolized by the gut microflora, and the resulting metabolites absorbed. Flavanols have a remarkable direct antioxidant activity, but a capacity to stimulate antioxidant enzymes has also been demonstrated. In addition, flavanols exert anti-inflammatory activities by inhibiting/lowering pro-inflammatory enzymes[152].

Many studies have focused on investigating the potential anti-inflammatory effects of flavanols, mainly epigallocatechin and proanthocyanidins from grape seeds, in animal models of IBD. In a first approach, an experimental diet containing catechin significantly decreased colonic damage and MPO activity in TNBS-induced UC in rats compared with a group fed a basal diet[153]. In an *in vitro* study, epicatechin was capable of inhibiting the permeabilization of Caco-2 cell monolayers induced after TNF-α treatment[154]. The preventive effects were mediated, at least in part, *via* inhibition of NADPH oxidase and NF-κB activation by reducing IκBα phosphorylation and subsequent nuclear transport and DNA binding. Similar protective results were obtained using catechin-7-O-β-D-glucopyranoside from *Phaseolus calcaratus* Roxburgh (fabaceae) seeds in a rat model of experimental colitis[155]. The treatment increased GSH levels and reduced MPO activity and protein levels as well as the mRNA and protein levels of lipid mediators (COX-2, iNOS, TNF-α, IL-1β) *via* inhibition the NF-κB pathway. Moreover, increased mRNA levels of the mucins MUC2 and MUC3, main components of the mucosal layer in the colon, were also evidenced.

Epigallocatechin-3-gallate (EGCG), a main constituent of green tea, has been extensively investigated as an anti-inflammatory agent in IBD. EGCG inhibited the gene expression and release of IL-8, PGE_2 and MIP-3α in human colon adenocarcinoma cell lines stimulated with TNF-α[156]. In diverse animal models of colitis, EGCG was evidenced to inhibit MPO activity and histamine levels in colon mucosa, to reduce macrophage chemotaxis and neutrophil infiltration, and to increase the activities of antioxidant enzymes and reduce the production of pro-inflammatory cytokines[157-159]. However, together with these beneficial anti-inflammatory effects, EGCG treatment has been reported to induce a macronutrient malabsorption which can represent a dose-limiting adverse effect that should be taken into account if it is to be translated to the clinic for treatment of IBD[160]. In this way, the co-administration of 1-piperoylpiperidin (piperine), an alkaloid with the capability enhancing EGCG availability, resulted in significantly higher anti-inflammatory effects,

with respect to EGCG alone, allowing the use of lower doses of EGCG (6.9 mg/kg body weight)[158]. Finally, the administration of peracetylated epigallocatechin-3-gallate (AcEGCG) was more active in preventing colon damage than EGCG[161]. The authors found that AcEGCG reduced inflammatory mediators by down-regulating the PI3K/Akt/NFκB pathway and increased the expression of heme-oxygenase-1 (HO-1) through induction of extracellular signal-regulated protein kinase (ERK)1/2 signaling together with acetylation of NF-E2-related factor 2 (Nrf2). Unfortunately, the mechanism of action was only investigated in AcEGCG and not in EGCG, making it difficult to know the cause of the different degree of activity between both compounds.

Proanthocyanidins from grape seeds were also investigated as therapeutic agents against UC in a rat model. Treatment with the proanthocyanidins significantly improved the colonic damage and decreased the pro-inflammatory mediators, such as MPO, as well as iNOS activity and levels of IL-1β and TNF-α, increased synthesis of the anti-inflammatory cytokines IL-2 and IL-4, reduced inflammatory cell infiltration, and increased antioxidant enzyme activities[162-164]. The reduction in mucosal inflammation was proposed to be mediated by inhibition of the NF-κB signal transduction pathway. The treatment with proanthocyanidins resulted in a significant reduction in IκB kinase (IκK) activation, leading to suppression in the phosphorylation-induced degradation of IκBα and nuclear translocation[163,165].

Protective effects of thearubigin and theaflavin-3,3'-digallate (TFDG), present in black tea, were evidenced as they ameliorated the disruption of colonic architecture and inflammation[166,167]. Similar to the other flavanols, the anti-inflammatory effects of thearubigin and TFDG seem to be mediated through down-regulation of the NF-κβ pathway by inhibiting the degradation of its endogenous inhibitor Iκβα.

Some complex mixtures rich in flavanols, such as polyphenol-enriched cocoa extract (containing catechin, epicatechin, procyanidin B1 and B2) and oligonol (containing 17.6% of catechin-type monomers and 18.6% of proanthocyanidins) were also effective against animal models of colitis[168,169]. Both treatments significantly reduced colon damage, inflammation, leukocyte infiltration and oxidative stress markers, whereas antioxidant enzyme activities were increased. The mechanism of action seems to involve the inhibition of transcription factors STAT1 and STAT3, which are associated with cytokine and growth factor receptors' synthesis and with innate and acquired immune cells' regulation, respectively. In addition, NF-κB activity was also inhibited, which indicates its participation in the anti-inflammatory effects of the extract.

CLINICAL TRIALS

Although there are multiple sources of evidence to support the anti-inflammatory effects of flavonoids, their therapeutic use in IBD has been almost exclusively studied in *in vitro* studies or animal models. To date, clinical studies are scarce, and further research with well-controlled procedures and higher number of patients is essential to establish the potential therapeutic use of flavonoids. In a first approach, an open pilot trial investigated the effect of anthocyanin-rich bilberry mixture in 13 subjects suffering from UC[128]. After 6 wk of intervention, elevated rates of clinical improvement were observed with a significant decrease in mucosal inflammation and a reduction in the levels of fecal calprotectin. However, after completion of the intervention, a raise in calprotectin levels and disease symptoms were reported following the 4-wk follow-up.

Another pilot study investigated the activity of (-)-EGCG (400 mg or 800 mg) that was supplied to 15 individuals with mild to moderate UC, whereas another 4 were assigned to a placebo control[170]. The response rate to the treatment after 56 d of therapy was 66.7% and the active treatment remission rate was 53.3%; on the contrary, none of the control subjects showed signs of improvement. Koláček *et al*[171] conducted a pilot study to determine the relation between oxidative stress in pediatric CD patients in remission and the influence of a polyphenol extract (Pycnogenol®, 70% ± 5% procyanidins). Patients reported reduced antioxidant defenses and increased oxidative damage markers when compared with healthy controls. A 10-wk course of polyphenol extract administration positively influenced the oxidative parameters in patients suffering from CD. A randomized, placebo-controlled clinical trial was designed to investigate the effects of silymarin (42 intervention *vs* 38 placebo) in UC patients[172]. Silymarin (140 mg) was given to the patients for 6 mo, together with the standard therapy. Silymarin was well tolerated by the patients and silymarin improved hemoglobin levels, ESR and disease activity index with respect to the placebo group.

CONCLUSION

It is well known that IBD causes prolonged inflammation of the gastrointestinal tract. However, the main cause of the IBD is unknown and currently there is no effective treatment to cure this disease. Anti-inflammatory agents are the first course of clinical action in IBD treatment, reducing inflammation of the digestive tract but also eliciting many side effects. Flavonoids are potent anti-inflammatory compounds that could be an interesting alternative in the IBD management. Diverse studies have reported significant beneficial effects of specific flavonoids or foods rich in these compounds against inflammation in IBD using animal models and cell culture.

Tables 1 and 2 summarize the effects of flavonoids in animal models of UC and CD. The immense majority of these investigations have been conducted

Table 1 Positive anticolitis effects of flavonoids on animal models of ulcerative colitis

Flavonoid	IBD model	Ref.
Quercetin	Acetic acid (mice)	[30]
Quercetin	DSS (rats)	[29]
Rutin	DSS (mice)	[29]
Kaempferol	DSS (mice)	[72]
Diadzein	DSS (mice)	[94]
Naringenin	DSS (mice)	[101]
Hesperidin	DSS (mice)	[104]
Anthocyanin (strawberry)	Acetic acid (rats)	[118]
Anthocyanin (blueberry)	Mdr1a$^{-/-}$ (mice)	[120]
Anthocyanin (cranberry)	DSS (mice)	[122]
Apigenin	DSS (mice)	[134]
Baicalein	DSS (mice)	[138]
Luteolin	DSS (mice)	[140]
Fisetin	DSS (mice)	[146]
Epigallocatechin-3-gallate	DSS (mice)	[158-161]
Oligonol	DSS (mice)	[169]

DSS: Dextran sulfate sodium; IBD: Inflammatory bowel disease.

Table 2 Positive anticolitis effects of flavonoids on animal models of Crohn's disease

Flavonoid	IBD model	Ref.
Quercetin	TNBS (rats)	[42]
Rutin	TNBS (rats)	[147]
Rutin	T cell transfer (mice)	[49]
Morin	TNBS (rats)	[52,53]
Genistein	TNBS (rats)	[83]
Diosmin	TNBS (rats)	[41]
Tangeretin	TNBS (mice)	[142]
Catechin	TNBS (rats)	[153]
Epigallocatechin-3-gallate	TNBS (rats)	[157]
Proanthocyanidins (grape)	TNBS (rats)	[162-165]
Thearubigin	TNBS (mice)	[167]

IBD: Inflammatory bowel disease; TNBS: Trinitrobenzene sulfonic acid.

in DSS and TNBS colitis models. More studies in other chronic models of IBD (*e.g.*, T cell transfer, IL-10 knockout, chronic oxazolone models of colitis) would be beneficial. Moreover, such studies could be conducted in conjunction with currently used IBD drugs (*e.g.,* mesalamine, corticosteroids), in order to probe for additive/synergistic pharmacological effects. Such studies may provide an impetus for designing/conducting future clinical trials with flavonoids as adjunct therapeutic agents.

Clinical trials analyzing the efficacy of flavonoids against IBD are still lacking and greater efforts should focus on studies with human patients. Further in-depth clinical studies are mandatory for confirming the therapeutic effects of flavonoids in IBD. More research within the fields of genetics, immunology, biochemistry and microbiology will provide a better knowledge of the underlying mechanisms involved in IBD, and are expected to increase treatment possibilities and their efficacies. Flavonoids could be a useful adjunct therapy in order to reduce or to ameliorate the symptoms of IBD and to potentiate the effects of future therapies.

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ORIGINAL ARTICLE

Basic Study

Myo-inositol reduces β-catenin activation in colitis

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Institutional review board statement: For all human studies, informed consent was obtained from every patient and samples were coded. Collection of all material was approved by the Northwestern University or University of Kentucky Institutional Review Boards. Some biopsies were collected under the NCI Myo-Inositol Chemoprevention in Colitis-Associated Dysplasia trial, #NWU09-13-02. The ClinicalTrials.gov identifier for the Myo-Inositol Chemoprevention trial is NCT01111292.

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Abstract

AIM

To assess dietary myo-inositol in reducing stem cell

activation in colitis, and validate pβ-catenin^{S552} as a biomarker of recurrent dysplasia.

METHODS

We examined the effects of dietary myo-inositol treatment on inflammation, pβ-catenin^{S552} and pAkt levels by histology and western blot in IL-10$^{-/-}$ and dextran sodium sulfate-treated colitic mice. Additionally, we assessed nuclear pβ-catenin^{S552} in patients treated with myo-inositol in a clinical trial, and in patients with and without a history of colitis-induced dysplasia.

RESULTS

In mice, pβ-catenin^{S552} staining faithfully reported the effects of myo-inositol in reducing inflammation and intestinal stem cell activation. In a pilot clinical trial of myo-inositol administration in patients with a history of low grade dysplasia (LGD), two patients had reduced numbers of intestinal stem cell activation compared to the placebo control patient. In humans, pβ-catenin^{S552} staining discriminated ulcerative colitis patients with a history of LGD from those with benign disease.

CONCLUSION

Enumerating crypts with increased numbers of pβ-catenin^{S552} - positive cells can be utilized as a biomarker in colitis-associated cancer chemoprevention trials.

Key words: Chemoprevention; Dysplasia; Biomarker; Stem cell; Colitis-associated cancer

Core tip: We report that dietary myo-inositol reduced inflammation and intestinal stem cell activation in both genetic and chemically-induced mouse models of colitis. In a limited clinical trial of colitis patients with a history of recurrent low grade dysplasia (LGD), myo-inositol reduced the number of intestinal crypts with activated stem cells. This study demonstrated for the first time that nuclear pβ-catenin^{S552} staining discriminates between patients with inflammation and those with a history of LGD, suggesting that nuclear pβ-catenin^{S552} staining may reflect local expansions of activated stem cells with neoplastic potential. Based on these data, we propose a more extensive clinical trial.

Bradford EM, Thompson CA, Goretsky T, Yang GY, Rodriguez LM, Li L, Barrett TA. Myo-inositol reduces β-catenin activation in colitis. *World J Gastroenterol* 2017; 23(28): 5115-5126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5115.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5115

INTRODUCTION

Chronic inflammation in ulcerative colitis (UC) and Crohn's disease (CD) contributes to an elevated risk of colitis-associated cancer (CAC)[1,2]. Patients with UC have an overall 11-fold relative risk of cancer and a 38-fold increased risk if they are diagnosed before age 30[1]. Similarly, patients with CD have an 18-fold increased risk of developing colorectal cancer[2]. In these diseases, chronic inflammation increases the risk of dysplasia through a variety of mechanisms. Oxidative stress, eicosanoid production and signaling through the cyclooxygenase/lipoxygenase pathways[3,4], apoptosis, altered metabolism, and signaling through phosphoinositide 3-kinase (PI3K) and Akt[5-7] all contribute to the transition from inflammation to dysplasia.

β-catenin translocation into the nucleus is a hallmark of colorectal cancer. Mutations in the genes encoding proteins in the phosphatase and tensin homolog (PTEN)/PI3K/Akt and Wnt/β-catenin pathways lead to colorectal cancer[8-11]. Mutations in adenomatous polyposis coli promote dysplasia and cancer by reducing β-catenin degradation and enhancing nuclear accumulation, detected by immunohistochemical staining. Additionally, Akt phosphorylation of β-catenin at serine 552 (pβ-cat) increases nuclear translocation of pβ-cat, upregulates TCF/LEF transcription, and increases *in vitro* tumor invasion[8]. Data from our group showed that mutations in PTEN enhance PI3K/Akt signaling and pβ-cat levels, leading to increased polyp formation[10]. These data suggest that alterations in PI3K and β-catenin signaling directly affect stem cell activation in dysplasia.

Under conditions of chronic inflammation, cells undergo neoplastic transformation after acquiring founder mutations. Volgelstein and others coined the term "field effect" to reflect the clonal expansion of mutated cells that cause areas of dysplasia to appear in the vicinity of sentinel areas of sporadic cancer or polyps[9,12]. In many cancers, including CAC, clonal fields of cells harboring DNA mutations surround visible dysplasia or carcinoma. Based on analysis of colitis-induced dysplasia, Brentnall and colleagues proposed that populations of intestinal epithelial cells[13] carrying p53 mutations spread to adjacent crypts by migrating over colonic surfaces[14]. Clevers and others suggest that the field effect originates in mutated intestinal stem cells[15,16]. We hypothesize that staining for nuclear pβ-cat provides a useful biomarker in identifying such a field effect.

Myo-inositol, a naturally abundant 6-carbon polyalcohol isolated from cereals (*e.g.*, rough coat of rice), and its derivatives regulate a variety of intracellular pathways. Inositol polyphosphates are important as second messengers in signal transduction, act as antioxidants, and mediate calcium regulation in membrane signaling. Nuclear inositol signaling may also play a role in DNA repair and chromatin remodeling[11,17-21]. Due to these effects, inositol has been tested in a wide range of human clinical trials, including cancer prevention, autism, and psychiatric disorders.

Lam *et al*[22] conducted a dose escalation trial of myo-inositol in smokers with bronchodysplasia. They

found that myo-inositol (9 g twice a day) reduced dysplasia, as well as Akt and phospho-ERK staining in dysplastic tissues[23]. Given our previous data linking PI3K signaling to β-catenin activation we considered the possibility that myo-inositol could reduce dysplasia by reducing ISC activation in colitis. However, before launching a large scale clinical trial, we wished to develop the means for detecting elevated numbers of activated ISC in colitis patients using pβ-cat staining. Additionally, we wished to determine whether this marker would serve as a reliable read-out of ISC chemoprevention.

MATERIALS AND METHODS

Animals

C57BL/6 (WT) and C57BL/6 IL-10-/- (IL-10$^{-/-}$) mice were purchased from Jackson Laboratories (Bar Harbor, ME, United States). Mice were maintained under specific pathogen free conditions at the University of Kentucky. All experiments were approved by the University of Kentucky IACUC.

Serum myo-inositol measurements

Serum myo-inositol was measured using an enzymatic spectrophotometric assay as previously described[24]. Briefly, blood was collected by cardiac perfusion into untreated 1.5 mL tubes, incubated at room temperature for 1 h, centrifuged, and serum was collected and stored at -80 ℃. Serum samples were treated with glucose oxidase and catalase (Calbiochem, San Diego, CA, United States), deproteinized with perchloric acid, and subsequently neutralized. Enzymatic determination of myo-inositol was done by reading the absorbance change at 546 nm from the reduction of Fe^{3+} - to Fe^{2+} -bathophenanthroline disulphonic acid (VWR, Radnor, PA, United States) from the NAD+-dependent oxidation of myo-inositol by myo-inositol dehydrogenase (Sigma, St.Louis, MO, United States). Myo-inositol concentration was determined from a calibration curve prepared with aqueous standards.

Induction of colitis in mice

Thirteen IL-10$^{-/-}$ mice were divided into three groups: control (IL-10$^{-/-}$), piroxicam-treated (IL-10$^{-/-}$ Px), and piroxicam-treated plus myo-inositol (IL-10$^{-/-}$ Px/myo-inositol). Piroxicam-containing chow was made by Harlan-Teklad (Madison, WI, United States). As previously described, 8-10 wk old IL-10$^{-/-}$ mice were fed chow containing 65 mg/250 g piroxicam for one week to synchronize colitis, followed by 85 mg/250 g piroxicam to induce colitis[25]. Mice were sacrificed 14 or 42 d later. For the group treated with myo-inositol, mice were treated with 1% (w/v) myo-inositol (Sigma) in the drinking water for one week prior to piroxicam treatment and for the duration of the experiment. For experiments of dextran sodium sulfate (DSS)-induced colitis, WT mice were treated with 2% (w/v) DSS (MP Biomedical, Santa Ana, CA, United States) for eight cycles. A single cycle of DSS consisted of 7 d of DSS in the drinking water followed by a recovery period of 14 d of regular water. Mice were treated with 1% myo-inositol during the recovery periods.

Disease activity indices

IL-10$^{-/-}$ mice undergoing treatment were scored daily or every other day using a standard disease activity index (DAI) based on diarrhea, fecal blood (Beckman Coulter SENSA Hemoccult Test), and percent weight loss as previously described[26]. Each criterion was assigned a score from 0 (no diarrhea, fecal blood or weight loss) to 4 (severe diarrhea, visible fecal blood, and up to 20% weight loss). The daily combined DAI score is the sum of each individual criterion.

Histological analysis

Tissues were fixed in 4% neutral buffered formalin overnight, processed through paraffin, sectioned at 5 μm, and stained for hematoxylin and eosin (H&E). For murine tissues, colitis scores were calculated based on a graded scale of inflammation (0-3), extent (0-3), regeneration (0-4), crypt damage (0-4) and percent involvement (1-4) as previously described[27]. The combined colitis score is the sum of the scores for inflammation, extent, and crypt damage/regeneration.

Immunohistochemistry

For human sections, standard of care biopsies were processed, stained and analyzed by the pathology departments of the University of Chicago or Northwestern University. Slides were stained for H&E, β-catenin, p53 or Ki67 and staining patterns were interpreted by staff pathologists. For laboratory staining of anti-pβ-catenin^{S522} (pβ-cat) and Ki67, paraffin sections were rehydrated through graded alcohols and antigen retrieval was performed using Target Retrieval Solution (Dako, Carpinteria, CA, United States), pH 6.0, in a decloaking chamber. Sections were incubated with anti-pβ-cat (provided by Linheng Li, Stowers) or anti-Ki67 (TEC-3, Dako) followed by anti-rabbit or anti-mouse peroxidase-labeled polymer (Dako). Sections were developed using 3,3'-diaminobenzidine (DAB) tetrahydrochloride chromagen (Dako) and counter-stained with hematoxylin.

Cell fractionation

IEC were isolated with EDTA and depleted of CD45 cells with sheep anti-rat IgG magnetic Dynabeads (Life Technologies, Grand Island, NY, United States) preloaded with rat anti-mouse CD45 antibody. For subcellular fractionation, all buffers contained ProteaseArrest ™ protease inhibitor cocktail (G-Biosciences), and phosphatase inhibitor cocktails I and II (Sigma) in 1:100 dilution. IEC were homogenized in Buffer I (50 mmol/L Tris-HCl pH 7.4, 100 mmol/L NaCl, 0.01% digitonin), lysates were passed through 26G needle,

then centrifuged at 4 ℃ for 10 min. Pellets were resuspended in Buffer II (50 mmol/L Tris-HCl pH 7.4, 2% Triton X100, 100 mmol/L NaCl) and incubated on ice for 30 min, then centrifuged as above. Pellets were dissolved in Buffer III (50 mmol/L Tris-HCl pH 7.4, 0.25% n-Dodecyl-D-maltoside, 100 mmol/L NaCl, 2 mmol/L $MgCl_2$) and incubated with 2U of Benzonase (Sigma) per 100 μL of lysate for 30 min at room temperature. After centrifugation the resulting supernatant was used as the nuclear fraction.

Immunoblotting

Proteins were transferred to Immobilon FL (Millipore) membrane by semi-dry transfer (Bio-Rad, Hercules, CA) and membranes were blocked in Pierce Protein-free T20 blocking buffer (Thermo, Rockford, IL, United States) for 1 h. Primary antibodies for anti-pβ-catenin^{S522} (pβ-cat, provided by Linheng Li, Stowers), pAkt^{S473} (Cell Signaling, Davers, MA, United States), and β-actin (Sigma) were diluted 1:1000 and incubated overnight at 4 ℃. HRP-conjugated secondary antibodies (KPL, Gaithersburg, MD) were used at 0.02 μg/mL and blots were developed using West Pico ECL reagent (Thermo, Rockford, IL, United States).

TOPFlash assay

NCM460 cells (normal derived colon mucosa) were received by a cell licensing agreement with INCELL Corporation (San Antonio, TX, United States), and were propagated in M3:10 medium with addition of the conditioned medium (30%) from previously cultured NCM460 cells. Cells were transfected with a reporter construct containing TCF/luc to evaluate β-catenin transcription[28]. Transfected cells were pre-treated for 3 h with 5 mmol/L myo-inositol and overnight with 5 mmol/L myo-inositol or 10 ng/mL of TNFα[29,30]. Luciferase was detected with Luciferase Reagent (Promega, Madison, WI, United States).

Human sample collection

Colon biopsies were obtained from adult patients undergoing diagnostic or surveillance colonoscopy or surgical resection at Northwestern University or the University of Chicago. For the NCI myo-inositol clinical trial, all patients were diagnosed as having colitis-induced low grade dysplasia (LGD). Informed consent was obtained from every patient and samples were coded. Collection of all material was approved by Northwestern University IRB and sample analysis was approved by the University of Kentucky IRB. Some biopsies were collected under the NCI Myo-Inositol Chemoprevention in Colitis-Associated Dysplasia trial, #NWU09-13-02.

NCI clinical trial

Three patients with LGD completed the 90-d clinical trial; 1 was given placebo and 2 were given myo-inositol. Colon biopsies were collected at the start of the study and again 90 d later. Participants were provided a 90 d supply of either maltodextrin (placebo) or myo-inositol in 9 g packets. Myo-inositol was manufactured by Tsuno Rice Fine Chemicals Co., Ltd (Wakayama, Japan) and filled into pouches by PharmOps, Inc (Phillipsburg, NJ, United States). Myo-inositol and the matching placebo were provided by NCI, DCP. For the first 14 d, participants dissolved one 9 g single-dose packet of study agent in 8 ounces of juice once daily. Following this initial period, participants escalated to 18 g/d taken in divided dose - one 9 g packet in the morning and one 9 g packet in the evening for the remaining 76 d. Patients were instructed to dose at approximately the same time each day, 2 h prior to eating. Pre- and post-study biopsies were assessed for general histological findings, IHC of p53 and Ki67, and patterns of nuclear pβ-cat.

Digital analysis

Slides were scanned using an Aperio ScanScope XT™ slide scanner and visualized using ImageScope v11. A modified nuclear quantitation algorithm was used to identify DAB-positive and DAB-negative epithelial nuclei. Slides were assessed for pβ-cat staining using the following three criteria; (1) the number of crypts containing 2, 3, 4 or 5 pβ-cat-positive cells; (2) the number of high powered fields of view containing 3 or more crypts, each containing 2 or more pβ-cat-positive cells (clustering or "high frequency" fields of view); and (3) the number of pβ-cat-positive cells per 100 IEC. For analysis of proliferation, numbers of Ki67-positive cells per 100 IEC were counted in high frequency fields of view as well as adjacent tissue.

Statistical analysis

All pairwise comparisons were made using a Student's *t*-test; $P < 0.05$ was considered statistically significant. Comparisons among multiple groups were made using a one-way ANOVA with post-hoc Tukey's test.

RESULTS

Treatment with myo-inositol reduces pβ-cat levels in murine colitis

Results from prior work indicate that myo-inositol inhibits PI3K and ERK signaling as evidenced by reduced p-Akt and p-ERK staining in dysplastic tissues[23]. Given our previous data linking PI3K signaling to β-catenin activation we considered the possibility that myo-inositol limits ISC activation in colitis[31]. The effects of myo-inositol on β-catenin activation were initially examined in the IL10$^{-/-}$ and DSS colitis models, which are known to progress from inflammation to CAC.

Initially, to establish the relationship between dietary intake and serum levels, myo-inositol levels was measured in untreated IL-10$^{-/-}$ mice given 1% w/v myo-inositol in their drinking water. Baseline myo-inositol concentrations in IL-10$^{-/-}$ mice were 2.0 ± 0.2 μmol/L,

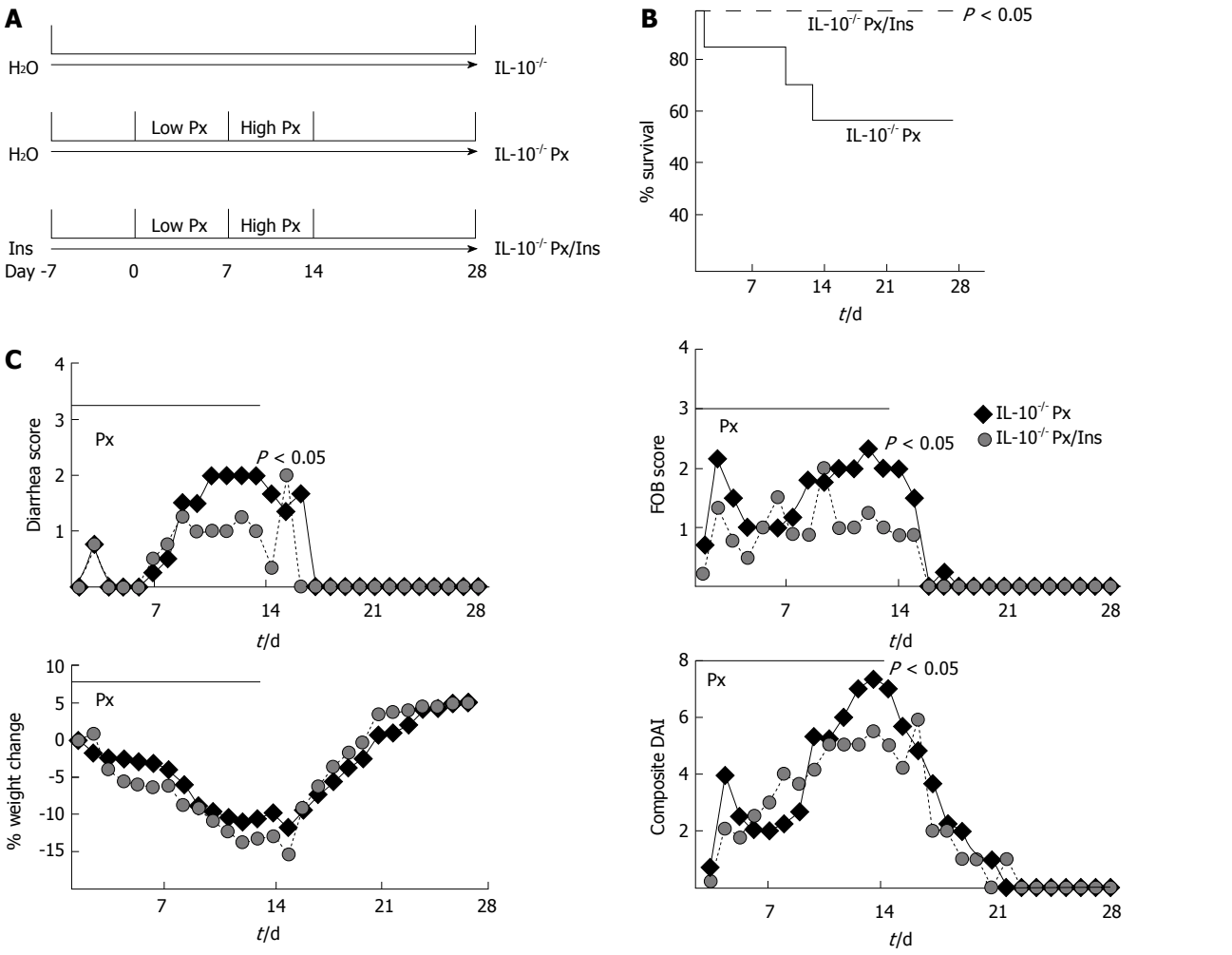

Figure 1 Myo-inositol reduces mortality and disease activity indices in IL-10$^{-/-}$ Px mice. A: Schematic diagram of Px and myo-inositol treatment in IL-10$^{-/-}$ mice; B: A Kaplan-Meier survival curve shows decreased mortality in IL-10$^{-/-}$ Px/myo-inositol mice; C: Disease activity indices (DAIs) for IL-10$^{-/-}$ Px and IL-10$^{-/-}$ Px/Ins mice. For diarrhea and fecal occult blood (FOB), scores are based on a scale from 0-4, with 0 being normal and 4 being the most severe (Methods). Percent weight change was calculated as weight change relative to the baseline weight at day 1. The composite DAI includes diarrhea and FOB scores, as well as weight change. The bar denotes Px treatment. Data points represent the mean, n = 4 mice in each group. P < 0.05 on day 14.

while provision of 1% myo-inositol in the drinking water raised serum myo-inositol concentrations 2.4-fold, to 4.9 ± 0.5 μmol/L, P = 0.02. Although our experiments were conducted with 1% myo-inositol, we tested the effect of 2% myo-inositol as well, and serum levels increased to 14.6 ± 1.1 μmol/L (P = 0.01 relative to 1% myo-inositol). These data document the correlation between dietary and serum levels of myo-inositol as used in these studies.

Piroxicam (Px) was used to induce colitis in IL-10$^{-/-}$ mice, and a cohort of these mice was treated with 1% myo-inositol in the drinking water (Figure 1A). While no IL-10$^{-/-}$ or IL-10$^{-/-}$ Px/myo-inositol mice died during the study, 42% of the IL-10$^{-/-}$ Px mice died during the second week of Px treatment (Figure 1B). Myo-inositol significantly reduced mean diarrhea scores in IL-10$^{-/-}$ Px mice from 2.3 ± 0.6 to 0.8 ± 0.3 (P = 0.02), and occult fecal blood scores from 2.0 ± 0.4 to 0.9 ± 0.3 (P = 0.02) (Figure 1C). There was no significant change in body weight between myo-inositol-treated and untreated mice, though myo-inositol may have contributed to increased caloric intake. Composite DAI scores were reduced by myo-inositol treatment (Figure 1C). Given that myo-inositol has anti-inflammatory effects[32], we next examined tissue histology in IL-10$^{-/-}$ Px and IL-10$^{-/-}$ Px/myo-inositol mice.

Tissue analysis revealed that myo-inositol significantly reduced inflammation in colonic tissue from IL-10$^{-/-}$ Px mice (Figure 2A and B). Images show that myo-inositol normalizes crypt distortion, transmural inflammation, and ulceration induced by Px in IL-10$^{-/-}$ mice. These changes correlate with increased myeloperoxidase (MPO) staining in IL-10$^{-/-}$ Px mice and greatly reduced MPO staining in IL-10$^{-/-}$ Px/myo-inositol mice (Figure 2A). This reduction was particularly evident near the junction of the proximal and distal colon, where IL-10$^{-/-}$ Px mice develop large lymphoid aggregates, ulcerated thickened mucosa, and crypt distortion. Histological grading of colitis (Methods) revealed that myo-inositol reduces inflammation scores, the extent of colitis, and composite colitis scores (Figure 2B). Inflammation scores were reduced

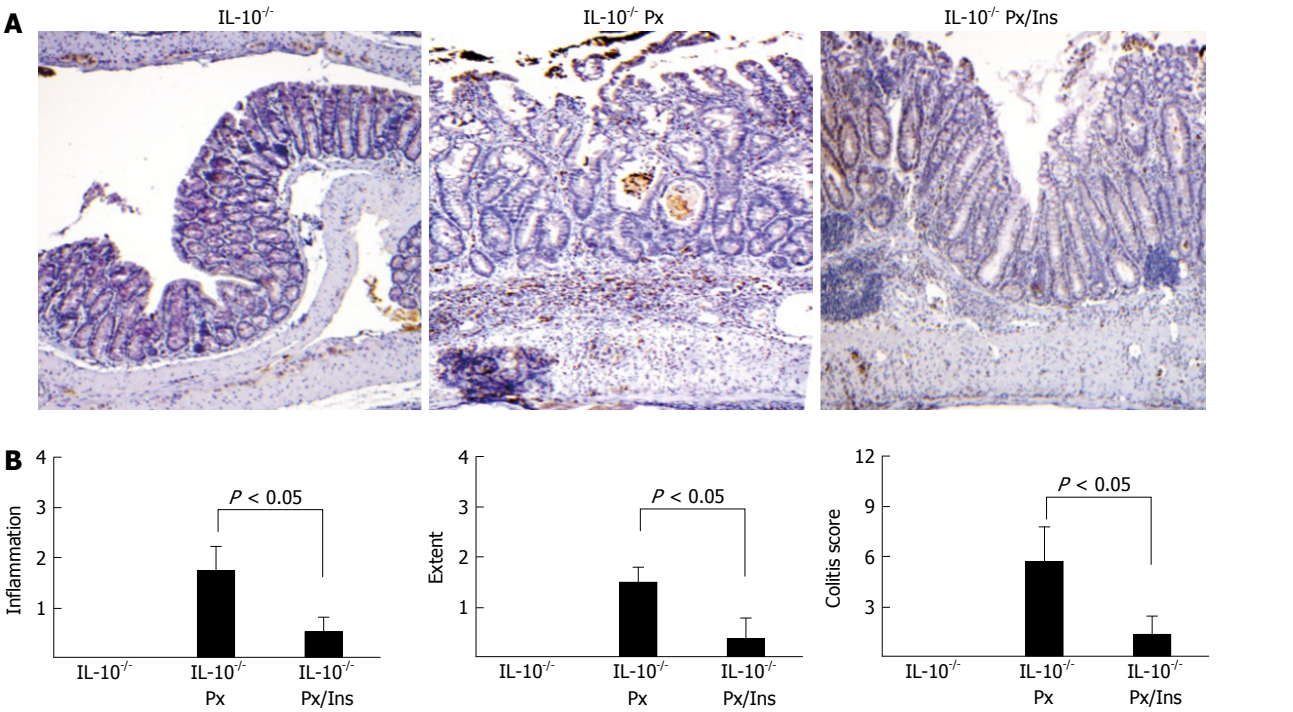

Figure 2 Myo-inositol reduces inflammation in IL-10$^{-/-}$ Px mice. A: Representative MPO staining of the proximal-distal junction in IL-10$^{-/-}$ control, IL-10$^{-/-}$ Px and IL-10$^{-/-}$ Px/myo-inositol colon at day 28; B: Histological grading of colitis shows that myo-inositol reduces the severity of inflammation, the extent (thickness) of inflammation, and the combined colitis score. Untreated IL-10$^{-/-}$ mice did not exhibit inflammation at day 28. Values represent the mean ± SE, *n* = 4 mice in each group.

in myo-inositol-treated IL-10$^{-/-}$ Px mice from 3.3 ± 1.9 to 1.0 ± 0.7 ($P < 0.05$) and myo-inositol completely abrogated crypt damage. Treatment with myo-inositol reduced the extent of colitis in IL-10$^{-/-}$ Px mice from 2.5 ± 1.2 to 0.5 ± 0.5 ($P < 0.05$).

pβ-cat staining can be used as a marker of activated stem and progenitor cells[25]. Thus, we examined the effect of myo-inositol on pβ-cat and pAkt levels in the IL-10$^{-/-}$mice (Figure 3A and B). Results show that myo-inositol abrogates colitis-induced pβ-cat induction. Cell counting revealed that the number of pβ-cat-positive nuclei dropped by 63% in myo-inositol-treated IL-10$^{-/-}$ Px mice from 0.3 ± 0.02 to 1.9 ± 0.02 ($P = 0.004$). Analysis of the crypt distribution of pβ-cat demonstrated that in IL-10$^{-/-}$ Px mice there were significantly more crypts with > 2 pβ-cat positive nuclei in mice with active colitis. By comparison, myo-inositol treatment normalized pβ-cat levels in IL-10$^{-/-}$/Px mice (Figure 3B). Similar effects of myo-inositol were observed with pAkt staining, in which myo-inositol reduced pAkt nuclear staining from 6.4 ± 2.0 to 2.3 ± 0.7 ($P < 0.05$) percent. Together these data suggest that myo-inositol impairs PI3K and β-catenin activation in colitis.

Previously we (GY) found that myo-inositol feeding reduced the incidence of cancer in a model of chronic DSS colitis[32]. To determine whether myo-inositol reduced levels of pβ-cat in these mice, we assessed protein levels of pβ-cat in isolated IEC from controls and mice treated with eight cycles of DSS with or without myo-inositol (Figure 3C). Levels of pAkt were assessed to determine if myo-inositol reduces this potential activator of b-catenin signaling. WB data show that myo-inositol reduced pβ-cat levels by 59%, from 45 ± 14-fold to 18 ± 4-fold, relative to DSS-treated controls ($P = 0.04$). Nuclear pAkt was reduced by 73%, from 11 ± 1-fold to 3 ± 1-fold ($P = 0.006$). Together, data from two colitis models indicate that oral myo-inositol reduces both pβ-cat and PI3K signaling in colitis.

To determine if myo-inositol can reduce inflammation-induced β-catenin activation, we used the *in vitro* TOPFlash assay to assess β-catenin activity in response to treatment with TNF and myo-inositol. Myo-inositol treatment alone had no discernible effect on baseline β-catenin activation. NCM cells treated with 10 ng/mL TNF showed a 60% increase in β-catenin activation (1.00 ± 0.08 *vs* 1.6 ± 0.12, $P < 0.05$), which was abrogated by co-treatment with 5 mmol/L myo-inositol (1.6 ± 0.12 *vs* 1.13 ± 0.09, $P < 0.05$; Figure 3D). These findings provide evidence that myo-inositol directly impacts Wnt/β-cat signaling.

Effect of myo-inositol on ISC activation in patients with a history of LGD

Several studies, in both human and mice, have shown the ability of myo-inositol to reduce dysplasia[23,32]. Given the chemopreventive effects of myo-inositol on CAC in mice[32] and the inhibition of β-catenin and PI3K signaling reported here, we postulated that myo-inositol may also reduce ISC activation and CAC in patients with longstanding colitis and a history

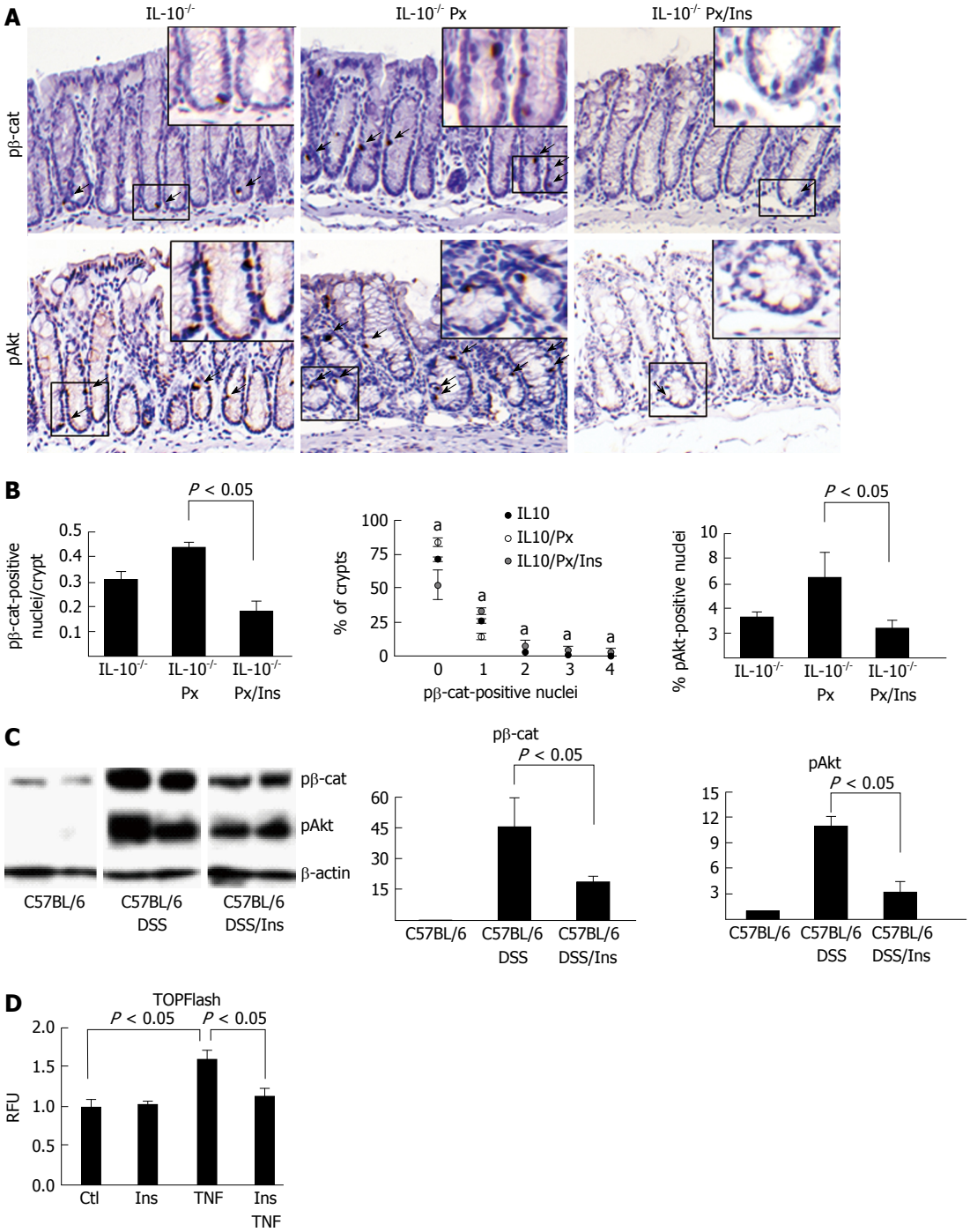

Figure 3 pβ-cat and pAkt are reduced in colitis mice treated with myo-inositol. A: Representative images of pβ-cat and pAkt staining; B: Quantification of the number of pβ-cat- and pAkt-positive nuclei. Distribution of the percent of crypts containing 0-5 pβ-cat-positive nuclei. Values represent the mean ± SE, *n* = 4 mice in each group. [a]*P* < 0.05; C: Western blots of IEC showing levels of nuclear pβ-cat and pAkt in the 8 cycle model of DSS-induced colitis. Lanes were rearranged to be consistent with formatting. Densitometry was normalized to β-actin and presented as the fold change (mean ± SE). *n* = 2 control mice, 3 DSS-treated mice, and 3 DSS/myo-inositol-treated mice; D: TOPFlash reporter assay (shown in relative fluorescence units, RFU) for β-catenin activation in NCM460 cells treated with TNF or myo-inositol. *n* = 4 separate experiments.

of recurrent dysplasia. To examine whether myo-inositol can be chemopreventive in CAC, we proposed a 3 mo trial in which myo-inositol was given to UC patients with LGD. We proposed that the frequency of pβ-cat-positive IEC would decrease in response to dietary myo-inositol treatment. Due to the challenges of finding colitis patients with current LGD at the time of consent, we were able to enter only five patients into the trial. One patient dropped out due to adverse events (diarrhea, abdominal pain, flatulence, hemorrhoids and increased serum bilirubin), though it is not clear that these were study-related. One patient had the area of LGD removed with the initial biopsy, precluding further analysis at the completion of the study. Three patients completed the study; 1 treated with placebo and 2 treated with myo-inositol. Statistical

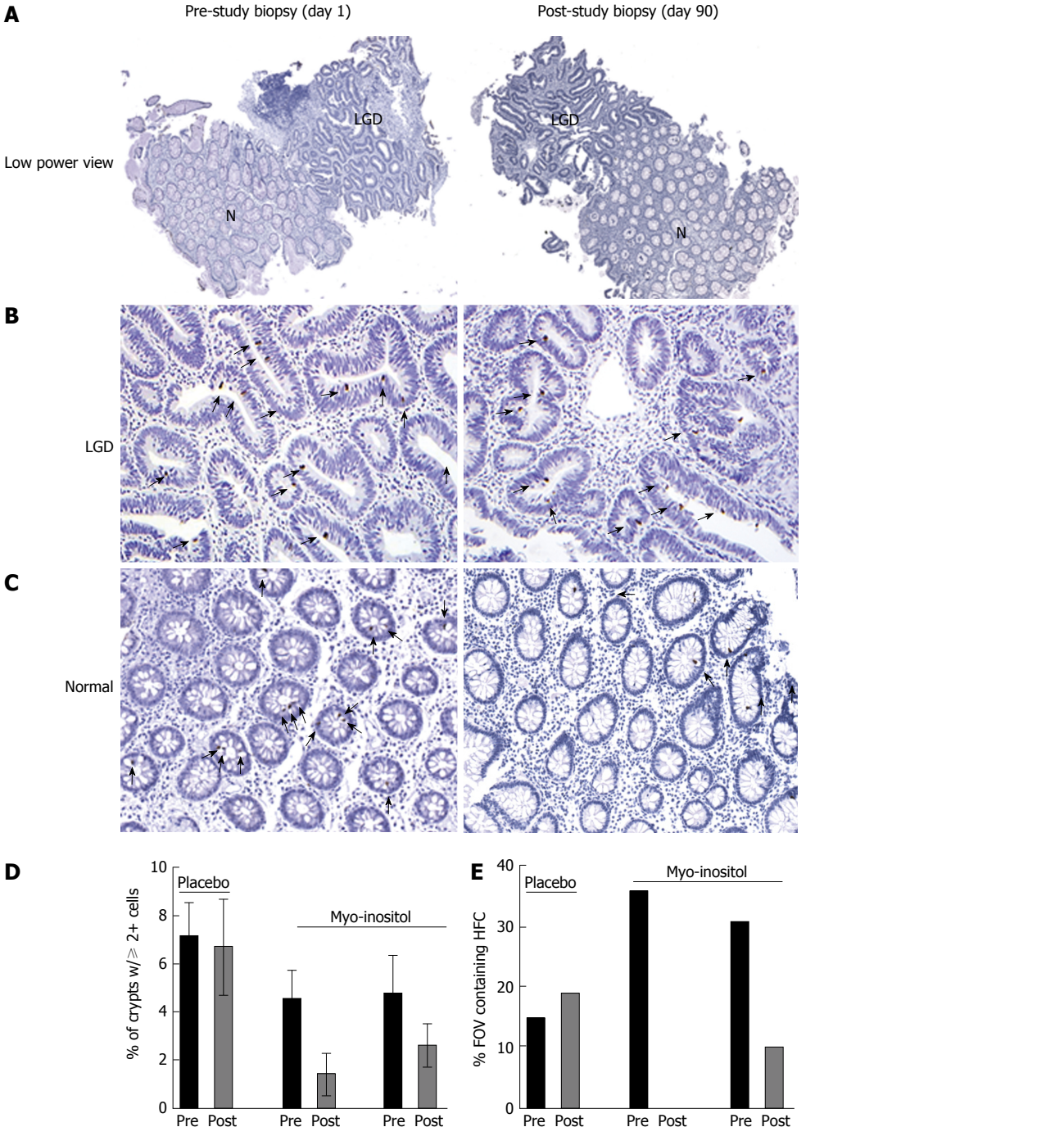

Figure 4 Myo-inositol reduces the number of pβ-cat-positive cells in ulcerative colitis patients. A: Representative low power images of biopsies stained for pβ-cat from a patient before and after 90 d of myo-inositol treatment. B: pβ-cat staining in an area of LGD; C: pβ-cat staining in a non-dysplastic area adjacent to LGD. Note the HFC containing multiple pβ-cat-positive nuclei; D: Graphic representation of the percent of crypts from each patient pre- and post-treatment containing 2 or more pβ-cat-positive nuclei; E: The percent of all high powered fields of view (FOV) containing HFC in biopsies from each patient. N: Normal tissue; LGD: Low grade dysplasia.

analysis of this small study size is not possible. However, the results of the staining were used to identify a trend of myo-inositol-induced reductions in the number of crypts with high numbers of pβ-cat-positive intestinal epithelial cells.

Data from the myo-inositol-treated patients who completed the study did not show reductions in established dysplasia (as in Figure 4A and B). However, in myo-inositol-treated patients there was a global reduction in the percentage of crypts containing two or more pβ-cat-positive cells in areas adjacent to LGD, which was not observed in the placebo-treated patient (Figure 4C). While analyzing the data from the 3 patients in the study, we observed "clusters" of crypts containing 2 or more pβ-cat-positive cells in non-dysplastic areas where pβ-cat staining was otherwise very sparse (Figure 4C). This aggregation of crypts with higher numbers of pβ-cat-positive cells is suggestive

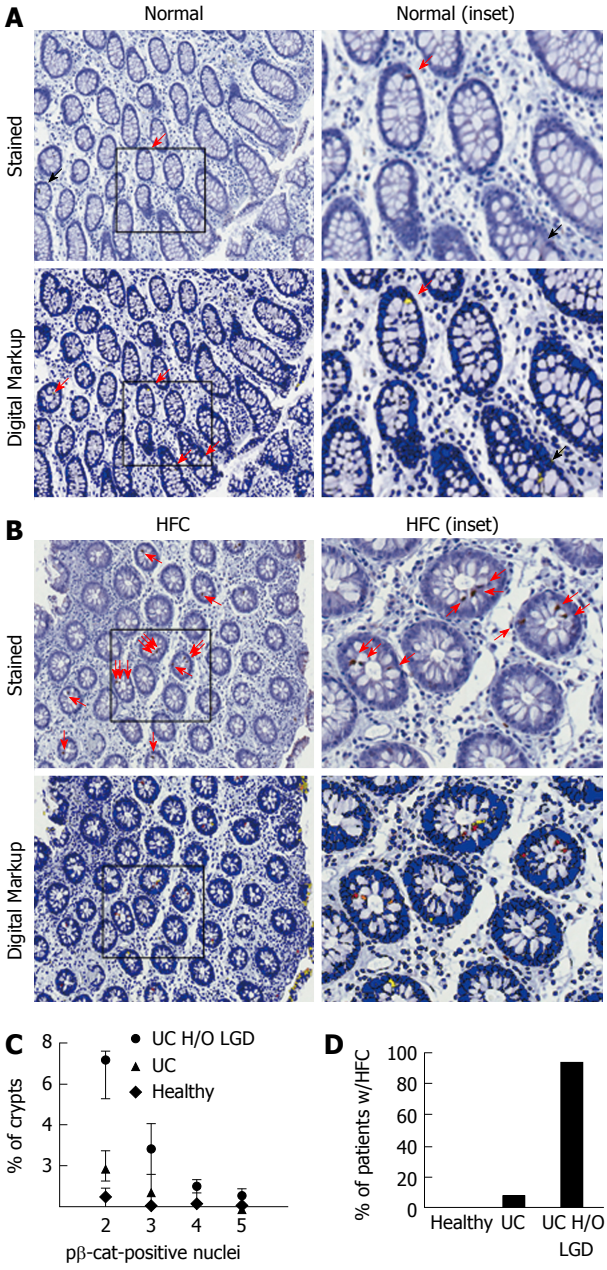

Figure 5 Crypts with elevated pβ-cat staining identify ulcerative colitis patients at a high risk for low grade dysplasia. A: Representative images of pβ-cat staining and the digital markup in colon biopsies of UC patients with normal pβ-cat distribution (benign disease); and B: HFC. Red arrows indicated pβ-cat-positive nuclei identified by eye; black arrows indicate false positive or negative nuclei properly identified by digital analysis; C: Statistical distribution of pβ-cat-positive nuclei in normal biopsies showing that crypts with 2 more positive pβ-cat-positive nuclei are rarely detected in healthy patients (diamonds, *n* = 3) or patients with benign, active UC (triangles, *n* = 12) but are more frequent in UC patients with a previous history of LGD (circles, *n* = 16). Data are shown as the percent of crypt cross-sections containing pβ-cat-positive nuclei; D: Biopsies from healthy controls, benign UC, and UC patients with a history of LGD were analyzed for the presence of HFC. UC: Ulcerative colitis; LGD: Low grade dysplasia; HFC: High frequency crypts.

of a field effect, in which foci of activated ISC are produced by expansion of mutated cells[14]. In order to quantify this effect, we counted the percentage of high power fields of view (20X) in which there were at least 3 crypts containing 2 or more pβ-cat-positive cells, henceforth defined as high frequency crypts (HFC). In the placebo-treated patient, the percent of HFC at day 1 and day 90 was 15% and 19%, respectively. However, in the patients treated with myo-inositol, the percentages of HFC at day 1 were 36% and 31%, which were reduced to 0% and 10%, respectively, by day 90 (Figure 4D). Although these findings are far from conclusive, they support the notion that myo-inositol reduces the frequency of activated stem cells in patients at high risk for CAC.

Objective assessment of pβ-cat nuclear accumulation in colitis

We previously observed that numbers of pβ-cat-positive IEC are increased in patients within areas of colitis-induced LGD and cancer, and inflammation[25]. However, the observation of pβ-cat-positive cell clustering within non-dysplastic tissue adjacent to areas of colitis-induced dysplasia led us to speculate that patients with a history of recurrent LGD could be stratified based on the presence of HFC. To test this hypothesis, a digital algorithm was developed to objectively distinguish pβ-cat-positive and negative nuclei in human biopsies. This allowed us to more accurately compare staining of tissues that were processed and stained by a variety of institutions and laboratory personnel.

Digital analysis of sections improved the quality of data generated by discriminating true positive and true negative cells (Figure 5A and B). In some cases, digital imaging identified positive cells that were not detected by trained observers (Figure 5A, black arrow). In other sections, the computer algorithm correctly discriminated false positives. The ability to accurately identify positive and negative nuclei significantly increased the sensitivity of pβ-cat staining.

To test the hypothesis that HFC could be used to stratify patients with a history of LGD, biopsies from healthy control patients (*n* = 3), patients with a diagnosis of active UC (*n* = 12), and UC patients with a history of LGD (*n* = 16) were stained for pβ-cat. In patients with a history of LGD, biopsies were taken from histologically normal areas of prior LGD, usually near mucosal tattoos marking resected LGD. In these tissues, we assessed the number of pβ-cat-positive cells within individual crypts and portrayed the relative distribution of crypts with distinct numbers of pβ-cat-positive cells/crypt (*e.g.*, percentage of total crypts with 2,3,4 or 5 pβ-cat-positive cells/crypt) (Figure 5C). In order to stratify patients, histologically normal tissues from areas of previous dysplasia were evaluated for HFC (Figure 5D). When we calculated the percentage of patients that had at least one HFC, 8.3% (1 out of 12) patients with benign, active UC had HFC whereas 94% of patients with a history of LGD had at least one HFC (15 out of 16). These findings suggest that focal increases in pβ-cat staining identify patients at risk for dysplasia. If further validated in more extensive clinical trials, this biomarker may provide a

useful means for testing chemopreventive agents such as myo-inositol.

DISCUSSION

The data presented in this study suggest that myo-inositol is a valuable chemopreventive agent that abrogates aberrant activation of ISC during inflammation and reduces the transition to dysplasia. Additionally, these data demonstrate that pβ-cat staining can be used to identify HFC within "pre-dysplastic" regions in patients with a previous history of colitis-induced dysplasia. A central hypothesis addressed in these studies was that myo-inositol reduces intestinal stem cell activation, detected by reduced nuclear pβ-cat and pAkt staining. Previous studies indicate that inositol compounds have both anti-inflammatory and chemopreventive properties[22,23,32-35]. Data here show that myo-inositol treatment reduces Akt activation in colitis. Additionally, we show that myo-inositol reduces β-catenin signaling, a key step in ISC activation. Prior data from our group demonstrated that Akt cooperates with Wnt to activate β-catenin[25]. In the models tested here, it is possible that PI3K inhibition by myo-inositol impaired Wnt signaling *via* reduced β-cat activation.

Because we were interested in early effects of myo-inositol during inflammation-induced neoplastic transformation, we analyzed data from IL-10$^{-/-}$ mice 14 d after the induction of colitis, 4 wk before the onset of dysplasia. The data presented here demonstrate that myo-inositol reduced inflammation, Akt activation, and pβ-cat nuclear localization. Additionally, in experiments with WT mice on 8 cycles of DSS, analysis of proteins from isolated IEC revealed that myo-inositol reduced nuclear pβ-cat and pAkt. In the same model, we reported that myo-inositol reduced tumor incidence, tumor multiplicity, and tumor volume[32]. Based on these results we propose that myo-inositol may reduce CAC by decreasing the numbers of activated ISC that may harbor pro-oncogenic genetic alterations.

5-ASA, a common therapeutic used in the treatment of UC, is a potent oxygen radical scavenger and has anti-proliferative and pro-apoptotic effects. 5-ASA reduces both pAkt and pβ-cat nuclear localization in human biopsies and in the IL-10$^{-/-}$ Px model of CAC[36,37]. However, in areas of established dysplasia, 5-ASA did not reduce proliferation or staining for pβ-cat and pAkt. We propose that like 5-ASA, myo-inositol reduces Akt/β-catenin signaling in the early stages of dysplastic transformation but not once it is established. We posit that 5-ASA and myo-inositol reduce CAC both by reducing inflammation-induced Wnt signaling in activated ISC at risk for acquiring founder mutations.

To address the question of whether a reduction in pβ-cat staining could be a biomarker of "fields" of pre-dysplastic cells in patients, we analyzed colonic biopsies from UC patients with benign disease or a history of LGD. We detected more regions with a high frequency of pβ-cat -positive nuclei (HFC) in tissue from patients with a history of LGD compared to those with benign disease. Using the criterion that tissue sections must have high powered fields of view with three or more crypts containing two or more pβ-cat-positive IEC, we were able to distinguish sections from UC patients from those who had a history of LGD and those who did not. We hypothesize that these data reflect local expansions of activated ISC harboring mutations that affect Wnt/β-catenin signaling. Inflammation-induced carcinogenic genetic and epigenetic changes may transmit a survival advantage to select ISC populations. Thus, our observations suggest that higher numbers of activated ISC may be a biomarker for areas of tissue at higher risk for neoplastic transformation. What remains unclear is whether myo-inositol primarily affects ISC by reducing inflammation, pro-carcinogenic inflammatory insults and/or by altering critical ISC signaling pathways (*e.g.*, PI3K, Wnt/β-catenin) relevant to carcinogenesis. In unpublished studies, we found that enhanced nuclear staining of pβ-cat correlates highly with increased TCF4/LEF immunoprecipitiation and increased expression of downstream TCF4 target genes. We propose that reductions of β-catenin activity in stem cells by myo-inositol are an essential mechanism for its chemopreventive properties. Because dysplasia typically arises from expansion of clonal stem cells that harbor mutations in key oncogenic processes[9,38,39], reducing the numbers of activated stem cells in colitis would presumably lower the risk for generating neoplastic crypts. Additionally, myo-inositol is a potent anti-oxidant, and as such it may also reduce DNA damage caused by reactive oxygen species. We hypothesize that myo-inositol may exert chemopreventive properties through both of these mechanisms.

In order to test the hypothesis that myo-inositol can be used as a chemopreventive in CAC, we propose a revised clinical trial. In this trial, UC patients would be recruited who have history of recurrent LGD and evidence of elevated pβ-cat staining in areas adjacent to prior dysplasia. Myo-inositol would be given long-term (1 year or more) and the primary endpoints would include (1) a reduction in the recurrence of LGD; and (2) normalized or reduced pβ-cat staining in areas proximal to LGD. The primary goal would not be the reversal of LGD, but rather the prevention of stem cell activation in "high risk" areas of pre-dysplastic fields resulting in reduced emergence of new LGD. The goal would be to abort the progression to recurrent dysplasia in patients who have a history of recurrent disease. The clear benefit would be the provision of a safe, low cost agent for patients who are found to have recurrent LGD during surveillance colonoscopy for colitis.

COMMENTS

Background

Patients with inflammatory bowel disease (IBD) have an increased risk

of dysplasia and colon cancer due to chronic inflammation. Developing a biomarker and identifying areas of the colon that harbor the potential for neoplastic transformation prior to the onset of histologically-identifiable dysplasia is critical for early treatment and cancer prevention. Additionally, there is a need for inexpensive, accessible pharmaceuticals to prevent recurrent dysplasia and the progression to cancer in IBD patients.

Research frontiers

Colitis-associated cancer (CAC) is a significant concern for patients with chronic inflammatory diseases, such as IBD. Although largely preventable by stringent surveillance colonoscopy and endoscopic or surgical removal of dysplastic tissue, many patients with limited access to health care resources remain at high risk for developing cancer. Myo-inositol is a safe, affordable, accessible sugar alcohol with limited side-effects and an established history in many clinical trials. We posit that myo-inositol is an attractive treatment option for patients at high risk for recurrent colonic dysplasia.

Innovations and breakthroughs

This study demonstrated that nuclear staining patterns of Akt-phosphorylated β-catenin (pβ-catenin^{S552}) positively correlate with levels of inflammation and proliferation in both mouse and human models of intestinal inflammation and IBD. Additionally, nuclear staining patterns of pβ-catenin^{S552} can be used to stratify patients with benign colitis and those with a history of dysplasia. In mouse models of colitis, oral myo-inositol significantly reduced inflammation and numbers of activated stem cells. In a limited clinical trial, myo-inositol treatment did not reduce dysplasia, but successfully altered the pattern of pβ-catenin^{S552} staining in areas adjacent to dysplasia, suggesting a role for oral myo-inositol in limiting stem cell activation during colitis.

Applications

In order to further test the hypothesis that myo-inositol can be used as a chemopreventive in CAC, the authors propose a revised clinical trial. In this trial, ulcerative colitis patients would be recruited who have history of recurrent low grade dysplasia (LGD) and evidence of elevated pβ-cat staining in areas adjacent to prior dysplasia. Myo-inositol would be given long-term and primary endpoints would include a reduction in the recurrence of LGD and normalization of pβ-cat staining in areas proximal to LGD. The primary goal would be the prevention of stem cell activation in "high risk" areas of pre-dysplastic fields resulting in reduced emergence of new LGD. The clear benefit would be the provision of a safe, low cost agent for patients who are found to have recurrent LGD during surveillance colonoscopy for colitis.

Terminology

LGD: Mild architectural abnormalities characterized by basally-oriented nuclei, mild nuclear enlargement, and nuclear crowding.

Peer-review

This paper describe some important things about: pβ-cateninS552 staining faithfully reported the effects of myo-inositol in reducing inflammation and intestinal stem cell activation in mice.

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ORIGINAL ARTICLE

Basic Study

Involvement of CRF2 signaling in enterocyte differentiation

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Abstract

AIM

To determine the role of corticotropin releasing factor receptor (CRF2) in epithelial permeability and enterocyte cell differentiation.

METHODS

For this purpose, we used rat Sprague Dawley and various colon carcinoma cell lines (SW620, HCT8R,

HT-29 and Caco-2 cell lines). Expression of CRF2 protein was analyzed by fluorescent immunolabeling in normal rat colon and then by western blot in dissociated colonic epithelial cells and in the lysates of colon carcinoma cell lines or during the early differentiation of HT-29 cells (ten first days). To assess the impact of CRF2 signaling on colonic cell differentiation, HT-29 and Caco-2 cells were exposed to Urocortin 3 recombinant proteins (Ucn3, 100 nmol/L). In some experiments, cells were pre-exposed to the astressin 2b (A2b) a CRF2 antagonist in order to inhibit the action of Ucn3. Intestinal cell differentiation was first analyzed by functional assays: the trans-cellular permeability and the para-cellular permeability were determined by Dextran-FITC intake and measure of the transepithelial electrical resistance respectively. Morphological modifications associated to epithelial dysfunction were analyzed by confocal microscopy after fluorescent labeling of actin (phaloidin-TRITC) and intercellular adhesion proteins such as E-cadherin, p120ctn, occludin and ZO-1. The establishment of mature adherens junctions (AJ) was monitored by following the distribution of AJ proteins in lipid raft fractions, after separation of cell lysates on sucrose gradients. Finally, the mRNA and the protein expression levels of characteristic markers of intestinal epithelial cell (IEC) differentiation such as the transcriptional factor krüppel-like factor 4 (KLF4) or the dipeptidyl peptidase IV (DPPIV) were performed by RT-PCR and western blot respectively. The specific activities of DPPIV and alkaline phosphatase (AP) enzymes were determined by a colorimetric method.

RESULTS

CRF2 protein is preferentially expressed in undifferentiated epithelial cells from the crypts of colon and in human colon carcinoma cell lines. Furthermore, CRF2 expression is down regulated according to the kinetic of HT-29 cell differentiation. By performing functional assays, we found that Ucn3-induced CRF2 signaling alters both para- and trans-cellular permeability of differentiated HT-29 and Caco-2 cells. These effects are partly mediated by Ucn3-induced morphological changes associated with the disruption of mature AJ in HT-29 cells and tight junctions (TJ) in Caco-2 cells. Ucn3-mediated activation of CRF2 decreases mRNA and protein expression levels of KLF4 a transcription factor involved in IEC differentiation. This signaling is correlated to a down-regulation of key IEC markers such as DPPIV and AP, at both transcriptional and post-transcriptional levels.

CONCLUSION

Our findings suggest that CRF2 signaling could modulate IEC differentiation. These mechanisms could be relevant to the stress induced epithelial alterations found in inflammatory bowel diseases.

Key words: Urocortin3; Colon carcinoma cell lines; Intercellular adhesion complexes; Cell permeability; Epithelial cell differentiation; Corticotrophin releasing factor 2

Core tip: Stress is recognized to participate in the development and/or aggravation of gastrointestinal (GI) disorders mainly by altering intestinal epithelial functions. This occurs partly through the deregulation of neuromediator secretion, but their activity on enterocyte differentiation remains not totally understood. We found that expression of corticotropin releasing factor receptor 2 (CRF2), a stress ligand receptor is inversely correlated to the differentiation status of colon cell lines. We thus investigated the effect of CRF2 signaling on intestinal epithelial differentiation using Ucn3 agonist treatments applied to different cancer cell lines that mimic this process. We identified that CRF2 activation affect both the early steps of differentiation and an established differentiated state, by down-regulating transcription factors such as krüppel-like factor 4. This effect is correlated with alterations of epithelial permeability and cellular adhesion junctions. Our results argue that CFR2-induced alterations of enterocyte differentiation may contribute to stress-mediated barrier dysfunction and the development of GI disorders.

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INTRODUCTION

The gut is composed of various cell populations distributed in different layers. These cells contribute to the intestinal epithelial homeostasis and function by controlling epithelial permeability, intestinal secretory, motor and immune functions. The intestinal epithelium consists of a monolayer of cells, which borders the lumen of the digestive tract and acts as a barrier against antigenic stimuli of food, bacterial, viral and parasitic origin. This organ is therefore the target of permanent aggressions whose accumulations can lead to a chronic inflammatory response favoring the deregulation of the morphology and functions of the intestine as it was observed in pathological disorders such as inflammatory bowel disease (IBD)[1]. In the long run, IBD can induce further complications and increase the risk of developing other pathologies such as colorectal cancer (CRC)[2]. Enterocytes, which represent 80% of the cells forming this monolayer, are specialized cells characterized by structural features

including polarized morphology and a complex set of cell-cell and cell-extracellular matrix (ECM) junctions. Three types of junctional complexes mainly ensure cell-cell adhesion: (1) tight junctions (TJ) composed of transmembrane proteins (such as claudin, occludin), which are linked to actin cytoskeleton (CSK) *via* scaffold proteins like zona occludens (ZO); (2) adherens junctions (AJ) which comprise E-cadherin connected to actin CSK *via* catenin and regulated by p120 catenins (ctn); and (3) desmosomes[3,4] and p120ctn regulate AJ by controlling cadherin clustering, endocytosis and stability as well as actin CSK anchorage[5].

In epithelial cells, assembly of adhesion complexes occurs at the plasma membrane, where individual proteins and lipids are known to be restricted to apical and basolateral domains. Others and we have shown that lipid rafts (LR) are specialized subdomains, highly enriched in cholesterol and sphingolipids, which play a role in the spatial organization and function of AJ and TJ[6,7]. As well as having a structural role, adhesion complexes are also preferential sites for signal transduction which control multiple aspects of the cell's behavior, mainly proliferation and differentiation[8-10]. Thus alterations of these signaling platforms may alter the differentiation process during intestinal epithelial renewal as well as during tumor development (review by[11]). This has been particularly highlighted in the intestinal epithelium by manipulating E-cadherin function[12]. The expression of E-cadherin protein is decreased in invasive CRC, a process that correlates with the acquisition of a mesenchymal phenotype[13]. Although each adhesion complex has its own particular mechanism of formation, regulation and function, theyall interact with one another through an extensive communication and mutually influence each other's dynamics and signaling properties.

In the last decade, stress (from psychological or environmental origins) has been recognized to participate in the development and/or aggravation of gastrointestinal (GI) disorders such as IBD or CRC[14,15-19]. The effects of stress are mediated through the secretion of specific stress neuromediators, such as corticotropin releasing factor (CRF) or its analogs Urocortin 2 and 3 (Ucn2/3)[19]. These peptides act through the activation of corticotropin releasing factor receptors 1 and 2 (CRF1/CRF2), two class II G protein coupled receptors (GPCR) with different affinities[17]. Ucn3 binds exclusively to CRF2[20]. The expression of CRF receptors and ligands in the GI tract has been investigated in rodents and humans (for review[21]). In the colon, all the cells that compose the different layers of the intestinal mucosa mostly express these molecules indicating that the intestine is a target for stress signaling. CRF receptors are primarily coupled to Gαs and trigger cAMP formation *via* adenylyl cyclase activation[18]. This signaling pathway could participate in the dissociation of intercellular adhesion complexes in intestinal epithelial cells (IEC)[22]. CRF receptors are also able to activate the Src kinase by promoting its auto-phosphorylation on Y^{418}[23]. Activation of src kinase could contribute to the opening of the intestinal barrier by modulating the phosphorylation status of intercellular junction proteins[24]. We previously demonstrated that CRF2 activation signals through the Src/ERK pathway to modulate cell-cell junctions in CRC cell lines[25].

The digestive epithelium is a very dynamic tissue that is constantly renewed. Indeed, it is fully regenerated within 3-5 d under normal homeostasis and this process is even faster after injury. This renewal is carried out by the stem cells located at the bottom of the crypts[26]. They first divide and give rise to progenitors (transiently amplified cells), which occupy most of the crypt, and then undergo a final division before starting a maturation and terminal differentiation program into either absorptive enterocytes or the secretory lineages (goblet, entero-endocrine and paneth cells). Differentiation takes place as the cells migrate in cohort along the crypt-villus axis before dying by anoïkosis and finally exfoliated at the tip of the villi in the small intestine. The mechanisms that regulate cell proliferation in the crypt, migration and differentiation of progenitor cells are partially understood. It is recognized that these mechanisms are based on fine spatio-temporal regulation of many genes along the crypt-villus axis. This regulation involves transcription factors (Cdx2, Hox, HNF, GATA4, KLF4...) expressed under the control of growth factors, hormones, cytokines but also by cell-cell or cell-ECM interactions[27,28]. Similarly, reciprocal interactions between the epithelium and the mesenchyme are necessary for the morphogenetic and differentiation processes that occur during organogenesis and migration along the crypt-villus axis[29-31]. Furthermore, IEC cell renewal and differentiation may also respond to environmental conditions including luminal nutriments, GI hormones and more recently psychological stress such as maternal deprivation (MD)[32-34]. Indeed, the CRF receptor signaling induced by MD markedly altered the quantitative distribution of secretary cells (paneth and goblet cells) of the intestinal epithelium, which may contribute to the development of epithelial barrier defects.

To date, the role of stress and its mediators on enterocyte differentiation has not been investigated. In the present study, our aim was first to characterize the expression pattern of CRF2 in normal rat colon epithelial cells and in human colon carcinoma cell lines. This distribution led us to determine the role of CRF2 signaling in the modulation of epithelial cell permeability and enterocyte-like differentiation.

MATERIALS AND METHODS

Cell culture and animals

All cell lines used in this study are human colon cancer cells. SW620, HCT8, HT-29 and Caco-2 cells obtained

from the American Type Culture Collection (ATCC, Manassas, VA, United States) were cultured at 37 ℃ in a 5% CO_2 atmosphere in DMEM media containing 25 mmol/L glucose (Invitrogen, Cergy Pontoise, France) and supplemented with 10% fetal bovine serum (Invitrogen), 5% penicillin and streptomycin. The cells were harvested in phosphate-buffered saline (PBS) supplemented with 1 mmol/L EDTA and 0.05% trypsin.

Cell differentiation: HT-29 cell differentiation was initiated by replacing standard medium of 90% confluent adherent cells by glucose-free DMEM (InVitroGen) supplemented with 10% dialyzed fetal calf serum, 5 mmol/L galactose, 15 mmol/L HEPES pH 7, selenous acid (10^{-2} g/mL), penicillin, and streptomycin. This medium (Gal medium or differentiation medium) was changed every day. Caco-2 cells differentiate spontaneously when they reach confluence. The first day of confluence was determined by phase contrast microscopy and designated "day 0". The medium was changed every day.

Male Sprague Dawley rats, 250-300 g, were purchased from Charles River France. The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimated to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for on week prior to experimentation. They were sacrificed in agreement with local ethic committee policies, by 40 mg/kg IP injection of pentobarbital sodium and submitted to 200 mL intra-cardiac perfusion of NaCl 0.9%. Epithelial cells were isolated from proximal colon section as previously described by[35]. Briefly, the tissue section was tied up at one end, reverted and filled to distension with PBS prior to closing the remaining open end. The section is then incubated with shaking at 37 ℃ for 45 min in 2 mL of dissociating buffer (PBS containing 1 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.3) containing protease inhibitor cocktail (Roche, Meylan, France). Dissociated epithelial cells were collected after a sedimentation step of 60 min and then harvested by centrifugation at 1500 rpm at 4 ℃ for 5 min, snap frozen in liquid nitrogen, and stored at -80 ℃.

Immunofluorescence microscopy

Rat proximal colons were collected, fixed overnight in PFA 4%, sucrose 3% and cryoprotected 12 h in sucrose 30% until freezing in isopentane. Samples of 3 different rats were embedded in Tissue-Teck OCT compound, cryosectioned at 30 μm thickness and placed on Superfrost plus slides for immunofluorescence (same protocol as for cells - see below).

Cells grown on glass coverslips were fixed with 3% (wt:vol) paraformaldehyde, 2% sucrose for 10 min at 37 ℃, and further permeabilized with 0.5% Triton X-100 in PBS for 15 min at room temperature (RT). Cells were washed twice in PBS containing 0.05% Tween 20, and blocked with 3% bovin serum albumin diluted in washing buffer for 1 h at RT. Then cells were stained for 1 h at 37 ℃ with primary antibody diluted in blocking solution and then incubated with secondary antibody coupled to Alexa 488 (1:500) for 45 min at 37 ℃. Cells were washed three times in washing buffer after each incubation with antibodies. Before the last wash, nuclei were labelled during 5 min with TO-PRO3 (Invitrogen). Coverslips were permanently mounted with Mowiol (Calbiochem, France). Fluorescence photomicrographs were taken using a confocal laser-scanning microscope (Leica TCS SPE). Primary antibodies were used at the following dilutions: anti-CRF2 (1/200; Abcam 12964, Paris, France), anti- human E-cadherin (1:500; HECD1, Takara Biochemicals, Paris, France), anti-p120ctn (1:200, Beckton Dickinson, France), and phalloidin-TRITC (25 ng/mL) (Sigma-Aldrich, L'Isle d'Abeau, France).

Protein extraction and Western blot analysis

For total protein extraction, cells were lysed with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 2 mmol/L $CaCl_2$ and $MgCl_2$) supplemented with a mixture of protease inhibitors (1:100, Roche, Meylan, France). Protein concentrations in lysates were determined using the copper reduction/bicinchoninic acid (BCA) assay (Pierce Chemical Co) according to the manufacturer's instructions. Proteins (75 μg in Laemli buffer containing 2-mercaptoethanol) were resolved on 10% acrylamide gels, transferred onto activated PVDF membranes (Ge HealthCare, Dutscher, France). Membranes were first blocked in 5% fat-free dry milk, 0.1% Tween 20 in PBS for 2h at RT. After overnight incubation at 4 ℃ with primary antibodies diluted in the blocking solution, blots were washed 3 times in PBS, 0.1% Tween 20, and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (dilution of 1:30000, Jackson Immunoresearch, Immunotech, Marseille, France) for 1 h at RT before extensive washes. Immunoblottings were visualized by chemiluminescence (Amersham ECL reagents) and revealed on hyperfilm ECL (Ge Healthcare) and quantified with Image J software from NIH. Primary antibodies were used at the following dilutions: anti-CRF2 (1:1000), anti-villin (1:2000, gift of Dr. Robin from Institute Curie, Paris, France), anti-human E-cadherin (1:1000), anti-p120ctn (1:1000), anti-actin (1:100, Sigma-Aldrich), anti-DPPIV (1/1000, BML SA-451 Enzo Life Science) and anti-KLF4 (1/500, GENETEX).

Immunoblots shown are representative of at least three independent experiments. All graphs represent the mean value ± SEM of protein expression levels measured by densitometric analysis with "Image J" software (NIH). For quantification, the expression level of each protein was normalized to actin for each sample. In order to display a fold increase over control, the relative expression of proteins in control conditions was indexed to 1 for each of three repeated

experiments.

Functional permeability assays

Paracellular permeability (FITC-dextran intake): The paracellular permeability of early differentiated HT-29 or differentiated Caco-2 cell monolayers was assessed by determining FITC-dextran cell intake. Cells were seeded in 24-MultiWell dishes and allowed to differentiate 10 d in Gal medium. At day 10, 2 mL of medium containing FITC-dextran of 4 kDa (2 mg/mL, Sigma-Aldrich) and 10 mmol/L HEPES were put on the cultures. Subsequently, wells were treated with or without 100 nmol/L Ucn3 (Sigma-Aldrich) and cells were replaced in a 37 ℃, 5% CO_2 incubator. At scheduled times (10, 20, 30 and 40 min), FITC-dextran containing medium was removed and cells were washed twice in cold PBS before lysis in RIPA buffer. Measurements of fluorescence were performed using the microplate reader PHERAstar Plus (BMG LABTECH). Excitation wavelength: 485 nm and emission wavelength: 520 nm. The background is obtained by measurement of MH medium fluorescent. These experiments were realized at least three times.

Measurement of transepithelial electrical resistance (TEER): Cells were seeded in the top compartment of a 24-multiwell insert plate (Corning PET) with 0.4 μm pore size and cultured until they reached their differentiation state. In some experiments cells were pretreated overnight with 1 mmol/L Astressin 2b (A2b, Sigma-Alrich) before exposure or not to 100 nmol/L Ucn3. TEER was measured as described previously[36] using a Millicell Electrical Resistance System (Millipore, Bedford, MA, United States). TEER was calculated as ohm times centimeter squared (Ω. cm^2) by multiplying it by the surface area of the monolayer. The TEER of the polyester membrane in Transwells (approximately 30 Ω. cm^2) was subtracted from all readings.

Isolation of LR

Briefly, post-nuclear supernatant from HT-29 cells was solubilized in 1.5 mL of buffer A (25 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, 5 mmol/L EGTA, protease inhibitors mixture) containing 1% Triton × 100 for 1 h at 4 ℃. Lysates were ultracentrifuged after mixing with an equal volume of 80% sucrose in buffer A and laid down at the bottom of a ultracentrifuge tube, which was then overlaid with 6 mL of 38% sucrose followed by 3 mL of 5% sucrose, all diluted in buffer A. After centrifugation at 38000 rpm for 16 h in a SW41 rotor (Beckman Coulter) at 4 ℃, 1 mL fractions were harvested from the top.

RT-PCR and PCR

Total RNA extractions were performed using the Nucleospin RNA kit (Macherey-Nagel, France) according to the manufacturer's instructions. Two micrograms of total RNA was denaturized and subsequently processed for reverse transcription using the M-MLV (Euromedex, France) according to the manufacturer's instructions and then run on a thermocycler (Eppendorf). Primer sequences and probes are: KLF4 (Tm 56 ℃, 35 cycles), forward: TGCTGATTGTCTATTTTTGCGTTTA, reverse: GAGAAGAAACGAAGCCAAAACC; DPPIV(Tm 60 ℃, 35 cycles), forward: CCCGCGGCCTTTATAC, reverse: GTGGTAAGACGGAGCCTGAC; AP (Tm 67 ℃, 35 cycles), forward: GCAACCCTGCAACCCACCCAAGGAG, reverse: CCAGCATCCAGATGTCCCGGGAG; GAPDH (Tm 60 ℃, 25 cycles), forward: TCCTCCTGCGACAGTCA and reverse: CACCACCTTCTTGATGTCATC. PCR conditions were: 5 min at 92 ℃ followed by numbers of cycles depending on the primers (40 s at 92 ℃, 40 s at Tm and 1 min at 72 ℃) and 10 min at 72 ℃. PCR were analyzed on 1% (w/v) agarose gel. Quantification was performed using Image J (NIH software). GAPDH was used as housekeeping gene.

Enzymatic activity assays

Cells were suspended in lysis buffer (5 mmol/L Na_2SO_4, 1 mmol/L Tris/HCl, pH 7.6, supplemented with protease inhibitor cocktail) and sonicated for 30 s at 4 ℃ (400 J/W s). Then the homogenate was centrifuged for 10 min at 1060 *g*. The supernatant was collected for the enzymatic assays performed in microplate.

Alkaline phosphatase (AP) activity was measured with 1, 5 or 10 μL of lysates containing approximately 2.5 μg/μL of proteins adjusted to 100 μL with reaction buffer (50 mmol/L glycine pH 10.5, 0.5 mmol/L $MgCl_2$, 5 mmol/L $CaCl_2$ and 2 mmol/L $ZnCl_2$). Then 100 μL of substrate was added to each well (10 mmol/L p-nitrophenylphosphate disodium salt (Sigma-Aldrich) in reaction buffer). The plate was incubated for 2 h at 37 ℃ and stopped by the addition of 50 μL of 1 mol/L NaOH. The production of p-nitrophenol was estimated by measuring the optical density at 420 nm using the microplate reader PHERAstar Plus (BMG LABTECH).

Dipeptidyl peptidase activity was determined by the digestion of 50 μL of 3 mmol/L GlyPro-p-nitroanilide (Sigma-Aldrich) prepared in previously described reaction buffer by 50 μL of cell lysates for 30 min at 37 ℃. The reaction was stopped with 50 μL of 0.1 mol/L sodium acetate and reaction products were measured at 420 nm. Results are expressed as enzyme activity in international units per milligram of protein previously estimated by BCA assays (Pierce). All experiments were performed in triplicates.

Statistical analysis

The statistical methods of this study were reviewed by Jacques Brocard from the Grenoble Institute of Neurosciences (INSERMU1216). Statistics were performed using the Prism 5.0 software (GraphPad Software, CA, United States). Throughout the study, parametric 1way ANOVA tests followed by Bonferroni's multiple comparison tests for selected data have been performed as described. Note that for each kind of experiment (Functional permeability assays;

densitometric analyses and enzymatic activity assays), normality of distribution of all the measures was verified with a D'Agostino-Pearson omnibus normality test (not shown).

RESULTS

CRF2 expression and localization in colonic epithelial cells

CRF2 expression and localization in IEC is quite controversial (for review[21]). Confocal microscopy analyses performed on colonic section of Sprague-Dawley rats indicated that CRF2 is expressed at the basal membrane of epithelial cells present in the crypts (Figure 1A). CRF2 protein expression was further confirmed by immunoblotting on dissociated IEC (Figure 1B). CRF2 protein expression was also observed in various epithelial derived carcinoma cell lines (Figure 1C). However, our data indicated that CRF2 expression seems to depend on the differentiation status of the cell lines: CRF2 expression is higher in less differentiated cell lines such as SW620 or HCT8 compared to more differentiated cell lines such as HT-29 or Caco-2. Furthermore, the CRF2 expression is also inversely correlated to E-cadherin expression in these cell lines. These data have been confirmed by a correlation taking all values independently (r^2 = 0.8748, P < 0.0001). HT-29 and Caco-2 were able to differentiate under specific culture conditions. HT-29 cell differentiation is achieved by switching glucose with galactose in culture medium (Gal medium)[37]. During the first ten days of culture in Gal medium, which correspond to the initial step of HT-29 cell differentiation, cells undergo structural changes (polarity and development of mature AJ) and start to express some epithelial differentiation markers such as digestive enzymes [AP, dipeptidyl peptidase IV (DPP IV)…][38]. Acquisition of the epithelial phenotype takes several days, and the highest degree of differentiation has been found to be optimum after 50 d of culture in Gal medium. To further confirm the correlation between CRF2 expression and cell differentiation status, we investigated the level of CRF2 expression during the first ten days of HT-29 cell differentiation (Figure 1D). Changes in the expression of the villin protein (a calcium-regulated actin binding protein of the brush border of IEC) were used as control of HT-29 cell differentiation. Western blot analysis indicated that villin protein expression increased according to the kinetic of culture in the differentiating medium, while CRF2 protein expression decreased accordingly.

Taken together, our data suggest that CRF2 expression is associated to a poor differentiated status of IEC.

CRF2 signaling alters the trans- and para-cellular permeabilities

The presence of CRF2 at the basal membrane of colonic IEC and its expression regulation during differentiation of colonic cancer cell lines suggest that CRF2 agonist such as Ucn3 could act directly on IEC to regulate their differentiation status and their function. One important function of IEC such as enterocytes is to perform an effective barrier to harmful macromolecules and microorganisms while maintaining a selective permeability for nutriments. To face this challenge, IEC have developed two mechanisms: the management of ion selectivity, nutriments and solutes occurs *via* the para-cellular route crossing between the epithelial cells, while large molecules such as antigens and immunoglobulins pass through epithelial cells *via* the trans-cellular route[39]. We first analyzed the effect of CRF2 signaling on the trans-cellular permeability by measuring dextran-FITC (4 kDa) intake of early-differentiated HT-29 cells treated or not with 100 nmol/L Ucn3 during 5 h (Figure 2A). In control conditions the diffusion of dextran-FITC was stable during the time course of the experiment. In contrast, treatment with Ucn3 induced a two-fold increase in the release of dextran-FITC intake indicating that CRF2 signaling could increase the trans-cellular permeability (Two-way ANOVA, P < 0.01). Phase contrast microscopy indicated the presence of refractile structures (diameter comprised between 3 and 20 μm) in the cell monolayer following Ucn3 treatment (Figure 2B). These structures could be involved in the trans-cellular transport. To further investigate this hypothesis we labeled fibrillar actin with phalloidin-TRITC as a marker of intracellular trafficking vesicles (vacuoles). As shown in Figure 2C, treatment of early-differentiated HT-29 cells with Ucn3 (100 nmol/L, 2 h) favored the organization of intracellular spheres (their membranes were evidenced with phalloidin-TRITC) that co-localized with Differential Interference Contrast (DIC) signal.

We next investigated the impact of CRF2 signaling on para-cellular transport by measuring the TEER of ten days confluent HT-29 monolayers (Figure 3A). While HT-29 cells did not form mature TJ until a complete differentiation process (*e.g.*, at day 50 in Gal medium), we observed an increase of 70% in the TEER between undifferentiated and early-differentiated HT-29 cells suggesting that intercellular adhesion complexes involved in the permeability are functional (data not shown). At this stage, mature AJ complexes participate both in cell permeability and in the acquisition of TJ complexes since they are the first adhesion complexes to be formed[40]. In ten days confluent HT-29 monolayers, treatment by Ucn3 (100 nmol/L) induced a transient and statistically significant TEER decrease after 60 min that reverted within 300 min. This effect was strongly inhibited by Astressin 2b (A2b), a selective inhibitor of CRF2. As CRF2 signaling can modulate cell adhesion in adenocarcinoma cell lines[25], and since intercellular junctions are responsible of permeability, we investigated the effect of Ucn3 on the localization of E-cadherin and p120ctn, two major proteins of AJ complexes. Confocal microscopy analysis showed that E-cadherin staining

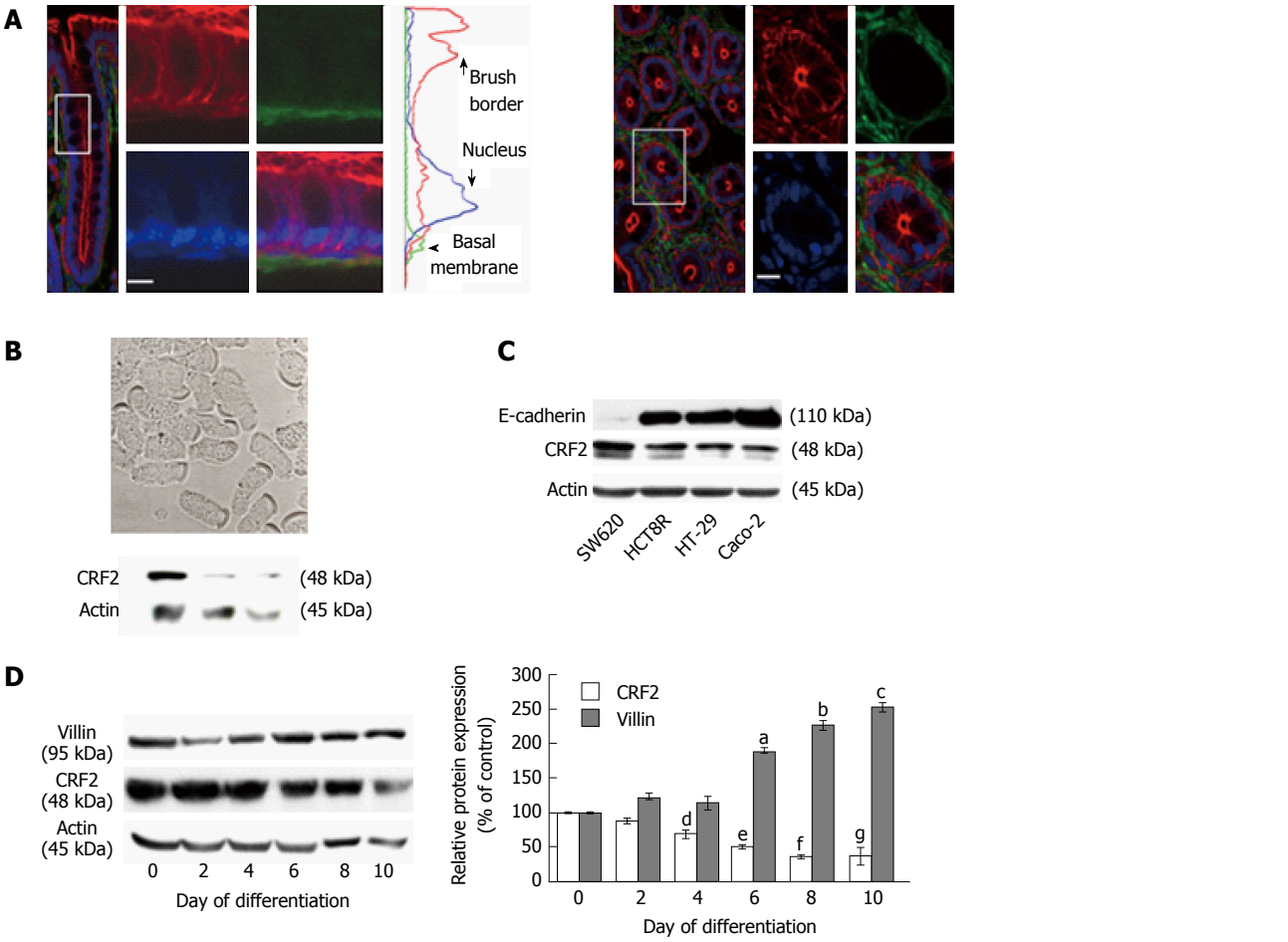

Figure 1 Corticotropin releasing factor receptor 2 expression in colonic epithelial cells. A: Confocal microscopy analysis of CRF2 protein expression (green) in Sprague-Dawley rat proximal colon. Actin and nuclei are labeled by phaloïdin-TRITC (red) and Topro-3 (blue). Longitudinal profile (left panel) and transversal profile (right panel) showing the crypts. Scale bar, 5 μm. Middle curves represent the means of fluorescence were measured according the basal-apical axis of epithelial cells. Acquisitions were performed on a Leica TCS SPE confocal microscope (objective × 100); B: Upper panel: Phase contrast analysis of dissociated epithelial cells from proximal colon of Sprague-Dawley rats. Scale bar, 5 μm. Lower panel: western blot detection of CRF2 expression in lysates of dissociated epithelial cells from three different animals; C: Detection of E-cadherin and CRF2 protein expression by western blot in various human colon carcinoma cell lines. Actin served as a loading control; D: Left panel: Detection of CRF2 and villin protein expression by western blot according to the kinetic of HT-29 cell differentiation. Actin served as a loading control. Right panel: Quantification of CRF2 and villin protein levels from western blot analysis. Data were expressed as fold increase of CRF2 or villin/actin protein levels of differentiated (D2, D4, D6, D8 and D10) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM, $^{a,b,c,e,f}P < 0.001$ *vs* undifferentiated HT-29 cells (D0), $^{d,g}P < 0.01$ *vs* D0. CRF2: Corticotropin releasing factor receptor 2.

appears as a honeycomb-like pattern at the membrane cortex (Figure 3B, left panel). Membrane E-cadherin staining is thickened and altered after 1 h and 2 h of treatment with Ucn3, while after 5 h the E-cadherin labeling seems to be restored. Similar results were observed with p120ctn staining, which is in part at the cell-cell contacts and some in the cytoplasm (Figure 3B, right panel). By stabilizing E-cadherin at the cell membrane, p120ctn participates in the regulation of E-cadherin function[41,42]. We have previously described that mature AJ required the recruitment of proteins in specific membrane domains called LR[6]. We thus examined the distribution of E-cadherin and p120ctn in LR identified by flotillin marker, after protein separation on sucrose gradient (Figure 3C). LR were purified from undifferentiated HT-29 cells or HT-29 cells differentiated ten days in Gal medium and then treated for 0, 2 h or 5 h with 100 nmol/L Ucn3. In all conditions tested, flotillin was found exclusively in fractions 3 and 4 (F3-4), which confirmed the presence of LR in those fractions. A small amount of flotillin was found in fractions 11-12 of undifferentiated HT-29 cells. In these cells, E-cadherin and p120ctn proteins are not expressed in LR, but distributed in fractions 5 to 12 with a stronger expression in fractions 9 to 12 (Figure 3C, upper panel). In ten days differentiated HT-29 cells, we found E-cadherin and p120ctn expressed in LR fractions at a level similar to fractions 5 to 8. The expression of these proteins was greater in fractions 9 to 12. After 2 h of treatment of differentiated HT-29 cells with Ucn3, we observed a decrease of E-cadherin and a loss of p120ctn expression in LR and fractions 5-6, while no changes were observed in fractions 9 to 12. Treatment for 5 h with Ucn3 induced a recruitment of E-cadherin and p120ctn proteins in LR compared to differentiated cells (fold increase of protein expression

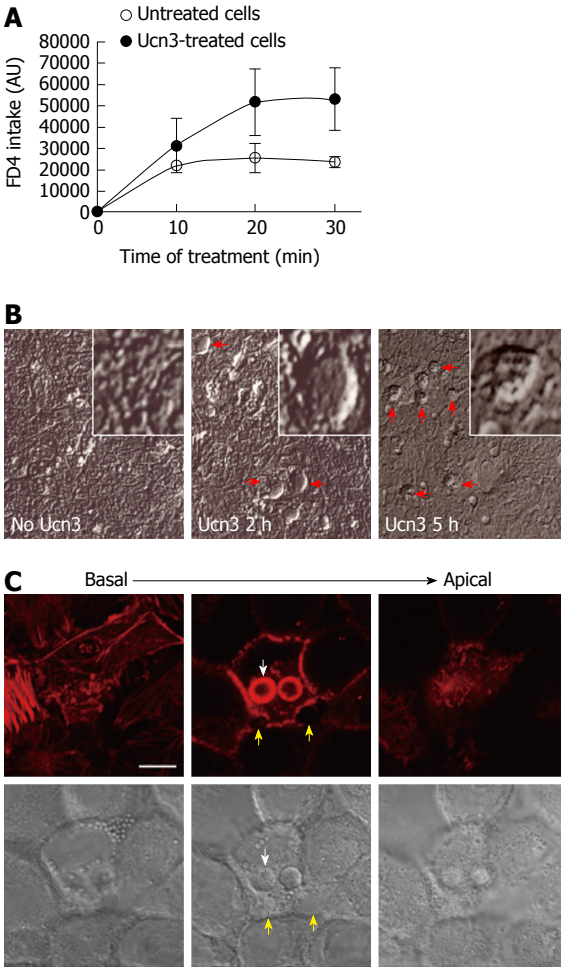

Figure 2 Corticotropin releasing factor receptor 2 signaling increases trans-epithelial permeability in early-differentiated HT-29 cells. A: The trans-cellular flux of dextran-FITC (4 kDa) was measured by spectrofluorimetry. HT-29 cells were cultured during 10 d in Gal medium. After 5 h treatment or not (untreated cells) with 100 nmol/L Ucn3, dextran-FITC was added at the apical compartment. Then the green fluorescence incorporated in the cell monolayer was measured after 10, 20, and 30 min. The release of fluorescence in the basal compartment is associated to trans-cellular permeability. Ucn3 treatment seems to increase transcellular transport in early-differentiated HT-29 cells. Two-way ANOVA, $P < 0.01$; B: Phase contrast analysis of early differentiated HT-29 cells exposed or not to Ucn3 (100 nmol/L). (Scale bar: 25 μm). Red arrows indicate the formation of refractile structures inside the epithelial monolayer; C: Confocal imaging from basal to apical poles of phaloidin-TRITC labeling (upper panel) and Differential Interference Contrast (lower panel) of early-differentiated HT-29 cells treated 2 h with 100 nmol/L Ucn3 (Scale bar: 10 μm). White arrows indicate refractile structures as observed before by phase contrast microscopy, while yellow arrows show alterations in cell-cell contacts. Acquisitions were performed on a Leica TCS SPE confocal microscope (objective × 100).

to flotillin in LR: 5.7 and 3.5 respectively) as well as in fractions 5 to 8. No differences in protein expression for E-cadherin was observed in fractions 9 to 12 while there was a slight decrease of p120ctn. The expression of TJ proteins (ZO-1, claudins, occludins…) had also been investigated in these gradients: their expression was restricted to fractions 9-12 and no modifications were observed in the different conditions studied (data not shown).

Interestingly, the recruitment of E-cadherin and p120ctn in LR after 5 h of treatment with Ucn3 correlated with the re-expression of AJ proteins at cell-cell contacts and the restoration of permeability efficiency. Taken together these results argue for a role of CRF2 signaling in the disorganization of AJ in early differentiated HT-29 cells which is probably responsible for the damage of para- and trans- cellular permeability.

CRF2 signaling delays colonic cell differentiation

Since Caco-2 cells differentiate spontaneously into enterocyte-like cells after reaching confluence, we used these cells to evaluate the effect of CRF2 signaling on IEC differentiation[43]. At 21 d post-confluence, Caco-2 cells are maximally differentiated into mature absorptive epithelial cells, both phenotypically and functionally. They form a polarized monolayer of cells that: express microvilli at their apical pole, contain digestive enzymes (such as disaccharidases or peptidases) and establish intercellular contacts with both mature AJ and TJ[44-46]. Therefore they constitute a good model for observation of passive para-cellular permeability[47] and analysis of IEC differentiation. Prior to exploring the effect of CRF2 signaling on cell differentiation we first checked the regulation of cell permeability by Ucn3 in differentiated Caco-2 cells (Figure 4A and B). Regarding the trans-cellular permeability, we found that as for HT-29 cells, the diffusion of dextran-FITC was stable during the time course of the experiment in untreated cells. However the barrier formed by Caco-2 cells was more stringent since a lesser intake of dextran-FITC is observed compare to HT-29 cells in the same conditions. Treatment with Ucn3 induced a 35-fold increase in the intake of dextran-FITC at 30 min indicating that CRF2 signaling could increase the trans-cellular permeability (Figure 4A, two-way ANOVA, $P < 0.001$). Compared to HT-29 cells, in absence of Ucn3, TEER is also higher in Caco-2 cells suggesting more efficient intercellular complexes. However, unlike HT-29 cells, Ucn3 treatment induced a transient decrease of TEER after 120 min that was not completely reverted within 300 min (Figure 4B). Nevertheless, A2b treatment totally abolished the Ucn3-mediated decrease of TEER confirming that CRF2 signaling alters intercellular junctions even in more differentiated epithelial cells such as Caco-2 cells. To confirm this hypothesis we examined the distribution of intercellular junction proteins by confocal microscopy (Figure 4C). We found that in 21-d differentiated Caco-2 cells, E-cadherin (AJ marker), occludin and ZO-1 (TJ markers) labeling was mostly at the cell membrane, at intercellular contacts. Following Ucn3 treatment (100 nmol/L, 2 h), the membrane labeling became punctuate and sometimes disappeared (occludin) whereas cytoplasmic labeling increased (E-cadherin and ZO-1).

Among the criteria of epithelial differentiation,

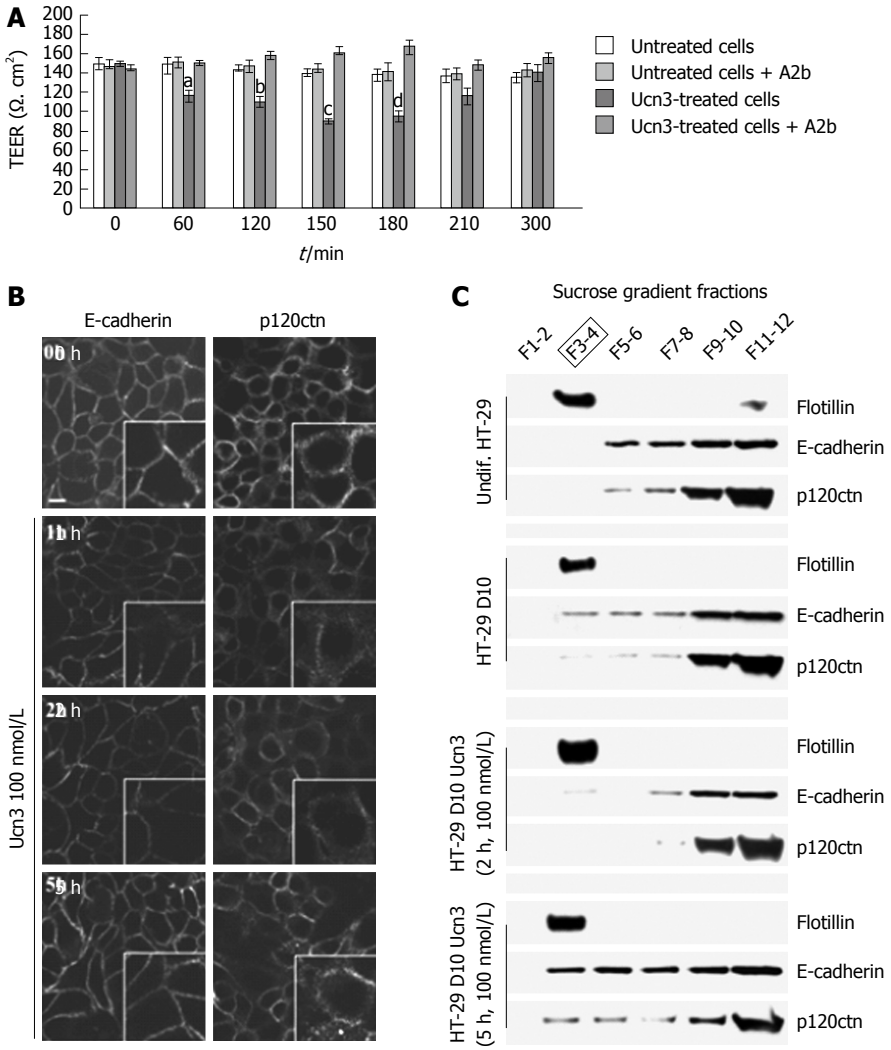

Figure 3 Corticotropin releasing factor receptor 2 signaling alters intercellular junction organization and increases para-cellular permeability in early-differentiated HT-29 cells. A: Early-differentiated HT-29 cells were pre-incubated with or without A2b (1 μmol/L) overnight before addition or not of Ucn3 (100 nmol/L). Intercellular junction integrity was evaluated by measuring transepithelial electrical resistance (TEER). Values are means of 5 different experiments ± SEM. $^{a,d}P < 0.05$ *vs* the three other groups, $^{b}P < 0.01$ *vs* the three other groups and $^{c}P < 0.001$ *vs* the three other groups; B: Early differentiated HT-29 cells were treated or not with 100 nmol/L Ucn3 before immunostaining for E-cadherin (left panels) and p120ctn (right panels). Bar is 5 μm. Images were acquired by confocal microscopy on a LEICA TCS/SPE (objective × 100). Ucn3 treatment induces a time-dependent alteration of AJ protein localization; C: AJ proteins are associated with Flotillin-1 enriched LR during HT-29 cell differentiation. Lysates from undifferentiated or early-differentiated HT-29 cells (10 d, D10) exposed or not to 100 nmol/L Ucn3 were fractionated using a sucrose gradient. The presence of LR was assessed by western blot against Flotillin-1. Western blot analysis of Flotillin-1, E-cadherin and p120ctn protein levels in LR under previously described conditions.

cell polarization and changes in gene expression are mostly reported. It has been previously described that intercellular complexes constitute a signaling platform involved in the regulation of cell differentiation by activating transcription factors responsible for cell growth arrest and differentiation marker expression[8,9,48,49]. Various transcription factors have been identified as regulators of IEC differentiation such as Cdx2, HNF4, GATA4 or Sox9. We focused our experiment on the Krüppel-like factor 4 (KLF4), a zinc-finger transcription factor that regulates a range of biological processes, including cell growth, differentiation and tumorigenesis[50,51]. Furthermore, KLF4 expression appears to be antagonist of CRF2 expression in normal intestinal epithelium as well as in CRC. Indeed, in normal intestine, expression of KLF4 is associated with the terminally differentiated state of epithelial cells near the luminal surface and of goblet cells in the crypts, whereas KLF4 is down-regulated in CRC[52-54]. It has also been shown to regulate IEC morphology and polarity and has been identified as a tumor suppressor[53,55-57]. To determine whether *in vitro* model of differentiation recapitulate the pattern of KLF4 expression suggesting a role in cell differentiation, we analyzed KLF4 mRNA and protein levels at different time points during 21 d of culture of Caco-2 cells (Figure 5A and B) or 10 d of culture of HT-29 cells (Figure 5C and D). KLF4 mRNA transcript levels increased according to the kinetic of differentiation and the maximal level of KLF4 mRNA transcript was detected at 21 d (2 fold increase compared to day 0) in Caco-2 cells and 10 d (3 fold increase compared to day 0) in

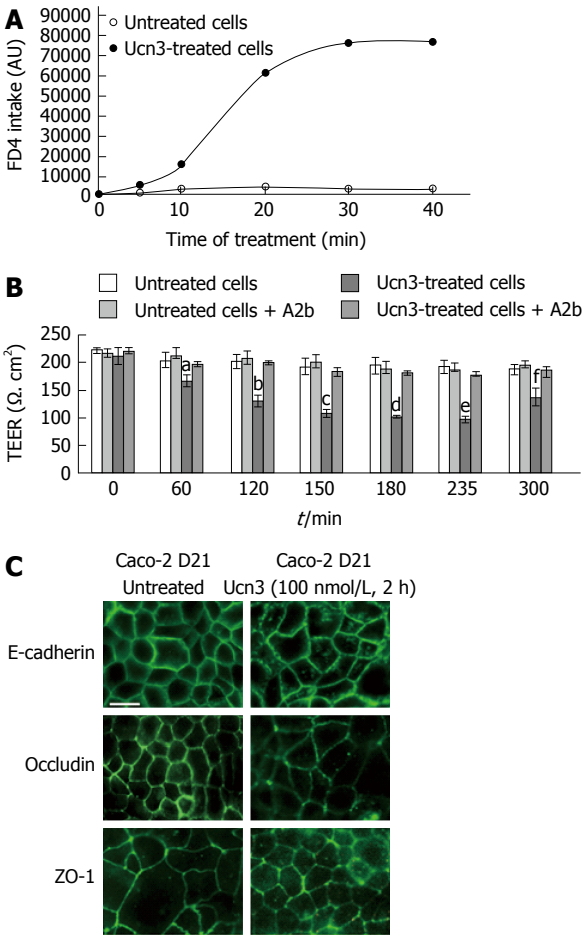

Figure 4 Corticotropin releasing factor receptor 2 signaling increases para-cellular permeability and alteration of adherens junctions and tight junctions in spontaneously differentiated Caco-2 cells. A: The trans-cellular flux of dextran-FITC (4 kDa) was measured by spectrofluorimetry. Caco-2 cells were cultured 21 d after they reached confluence. After 5 h treatment or not (untreated cells) with 100 nmol/L Ucn3, dextran-FITC was added at the apical compartment. Then the green fluorescence incorporated in the cell monolayer was measured after 5, 10, 20, 30 and 40 min. The release of fluorescence in the basal compartment is associated to trans-cellular permeability. Ucn3 treatment seems to increase transcellular transport in well-differentiated Caco-2 cells. Two-way ANOVA, $P < 0.001$. B: Twenty-one days differentiated Caco-2 cells were pre-incubated with or without A2b (1 μmol/L) overnight before addition or not of Ucn3 (100 nmol/L). Intercellular junction integrity was evaluated by measuring transepithelial electrical resistance (TEER). Values are means of 5 different experiments ± SEM. $^{a}P < 0.05$ *vs* the three other groups, $^{f}P < 0.01$ *vs* the three other groups and $^{b,c,d,e}P < 0.001$ *vs* the three other groups; C: Twenty-one days differentiated Caco-2 cells were treated or not with 100 nmol/L Ucn3 before immunostaining for E-cadherin (upper panels), occludin (middle panels) and ZO-1 (lower panels). Bar is 20 μm. Images were acquired by confocal microscopy on a LEICA TCS/SPE (objective × 100). Ucn3 treatment induces a time-dependent alteration of AJ and TJ protein localization.

HT-29 cells. However the basal level of KLF4 mRNA transcripts was greater in Caco-2 cells compared to HT-29 cells. We then analyzed the effect of Ucn3 on KLF4 mRNA transcripts in 21 d differentiated Caco-2 cells or 10 d differentiated HT-29 cells either exposed for 5 h at 100 nmol/L Ucn3 (acute treatment) or each day of differentiation with 100 nmol/L Ucn3 (chronic treatment). As shown in Figure 5A and C, Ucn3 completely abolished the differentiation mediated up-regulation of KLF4 mRNA transcripts following acute or chronic treatment. Regarding KLF4 protein levels, we found that KLF4 protein expression increased according to the kinetic of differentiation (Figure 5B and C); the maximal level of KLF4 protein was detected at 21 d of culture for Caco-2 cells (4.5 fold increase compared to day 0) and 10 d of culture for HT-29 cells (2 fold increase compared to day 0). Furthermore, in Caco-2 cells, Ucn3 reduced KLF4 protein enrichment at day 21 by 30% following acute treatment and totally abolished KLF4 protein enrichment following chronic treatment (Figure 5B). In HT-29 cells, Ucn3 totally abolished KLF4 protein enrichment at day 10 following acute and chronic treatments (Figure 5D).

Regulation of intestinal transcription factors has been correlated with the expression of several markers of mature epithelium at both the mRNA and protein levels. We previously observed that CRF2 expression is inversely correlated with villin during HT-29 cell differentiation (Figure 1E). We next tested the effect of CRF2 signaling on other characteristic markers of differentiated enterocytes, including dipeptidyl peptidase 4 (DPPIV) and the brush border enzyme AP. At the transcriptional level, we found that DPPIV and AP mRNA transcript levels increased according to the kinetic of differentiation of the both cell lines. The maximal level of DPPIV and AP mRNA transcript was detected at 21 d in Caco-2 cells (respectively: 10 fold and 6 fold increase compared to day 0) (Figure 6A, left panel). In HT-29 cells, the maximal level of DPPIV and AP mRNA transcripts was detected at 10 d (2 fold increase compared to day 0 for each transcripts) (Figure 6A, right panel). In Caco-2 cells, Ucn3 reduced DPPIV mRNA enrichment at day 21 by 50% following acute treatment and totally following chronic treatment. Acute treatment has very little effect on AP mRNA transcripts while chronic treatment reduced by 40% the level of AP mRNA transcripts. Once more, Ucn3 completely abolished the differentiation-mediated up-regulation of DPPIV and AP mRNA transcripts following acute or chronic treatment in HT-29 cells (Figure 6A, right panel). We next analyzed the effect of CRF2 signaling at the protein level in Caco-2 cells. We observed a marked increase of DPPIV protein expression, which coincided, with the kinetic of Caco-2 differentiation with a maximum at 21 d (6 fold increase compared to day 0) (Figure 6B). The expression of DPPIV protein in 21 d differentiated Caco-2 cells was highly inhibited with both acute (56%) and chronic (71%) exposure to Ucn3. Additionally, we looked at the specific enzymatic activities of DPPIV and AP. In line with the increase of DPPIV protein expression, we found an increase in the specific enzymatic activities of both DPPIV and AP during the time course of Caco-2 cell differentiation (Figure 6C and D). However, we observed that only chronic exposure to Ucn3 reduced both enzyme activities to their day 0 level, whereas acute treatment with Ucn3 had only a little effect on

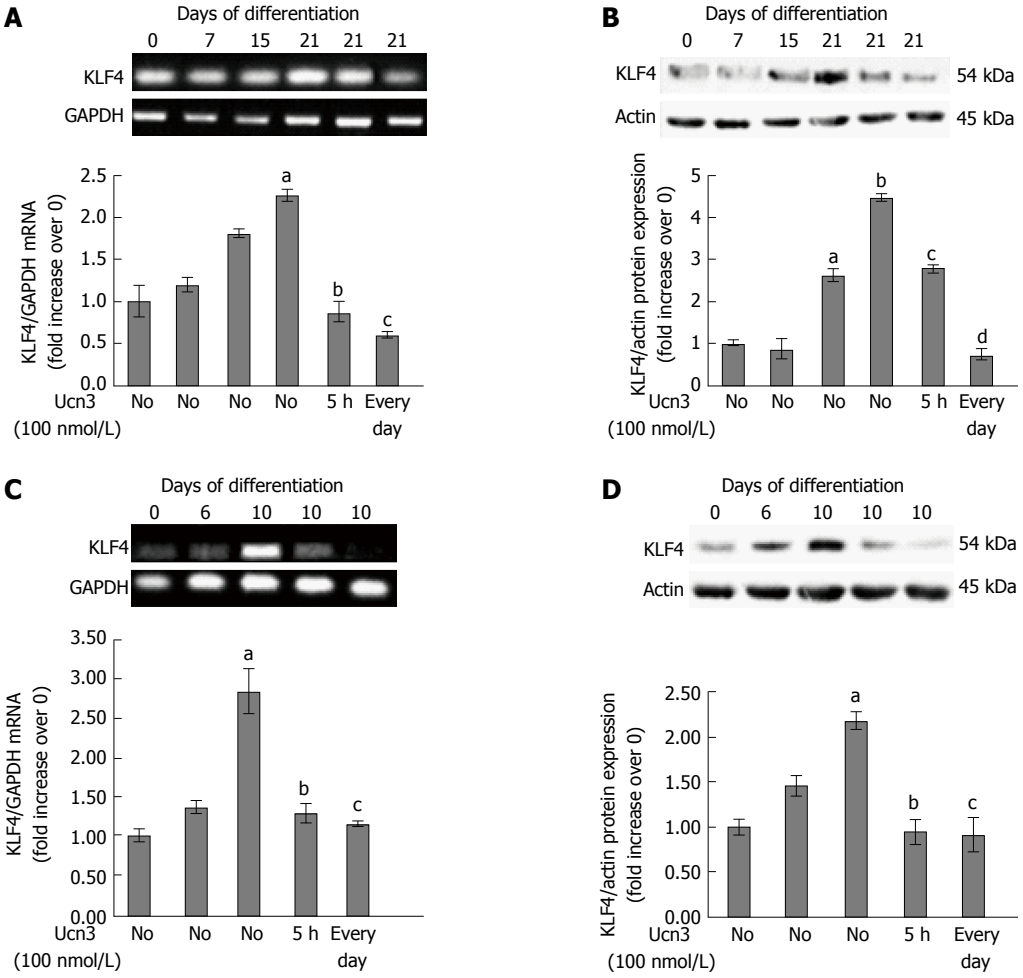

Figure 5 Down-regulation of KLF4 mRNA and protein expression following corticotropin releasing factor receptor 2 signaling. A: Detection of KLF4 mRNA expression by RT-PCR during the kinetic of Caco-2 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 21 d differentiated cells. GAPDH served as a housekeeping control. Quantification of KLF4 mRNA from RT-PCR assays (lower panel). Data were expressed as fold increase of KLF4/GAPDH mRNA levels of differentiated (D7, D15, D21) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. $^{a,b}P < 0.001$ *vs* undifferentiated Caco-2 cells (D0); $^{c}P < 0.001$ *vs* differentiated Caco-2 cells (D21). B: Detection of KLF4 protein expression by western blot during the kinetic of Caco-2 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 21 d differentiated cells. Actin served as a loading control. Lower panel: Quantification of KLF4 protein levels from western blot analyses. Data were expressed as fold increase of KLF4/actin protein levels of differentiated (D7, D15, D21) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. $^{a,b}P < 0.001$ *vs* undifferentiated Caco-2 cells (D0); $^{c,d}P < 0.001$ *vs* differentiated Caco-2 cells (D21). C: Detection of KLF4 mRNA expression by RT-PCR during the kinetic of HT-29 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 10 d differentiated cells. GAPDH served as a housekeeping control. Quantification of KLF4 mRNA from RT-PCR assays (lower panel). Data were expressed as fold increase of KLF4/GAPDH mRNA levels of differentiated (D6 and D10) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. Data represents means of three different experiments ± SEM. $^{a}P < 0.001$ *vs* undifferentiated HT-29 cells (D0); $^{b}P < 0.05$ *vs* early differentiated HT-29 cells (D10), $^{c}P < 0.01$ *vs* D10. D: Detection of KLF4 protein expression by western blot during the kinetic of HT-29 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 10 d differentiated cells. Actin served as a loading control. Lower panel: Quantification of KLF4 protein levels from western blot analyses. Data were expressed as fold increase of KLF4/actin protein levels of differentiated (D6 and D10) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. $^{a}P < 0.001$ *vs* undifferentiated HT-29 cells (D0); $^{b,c}P < 0.001$ *vs* early differentiated HT-29 cells (D10).

AP activity (Figure 6D, right panel).

Taken together these data indicate that CRF2 signaling may regulate IEC differentiation by modulating the expression of transcriptional factors involved in the regulation of characteristic markers of differentiated enterocytes.

DISCUSSION

In this study, we showed for the first time that CRF2 signaling might delay enterocyte differentiation either by affecting intercellular complexes but also by regulating gene and protein expression.

CRF2 expression in IEC and CRC cells

The CRFergic system is a central element of stress response. The expression and regulation of CRF2 have been mainly described at the level of the enteric nervous system (ENS), the enteric blood vessels and the immune cells of the mucosa[58]. Nevertheless, studies have demonstrated its expression in the IEC, particularly those localized in the upper region of the

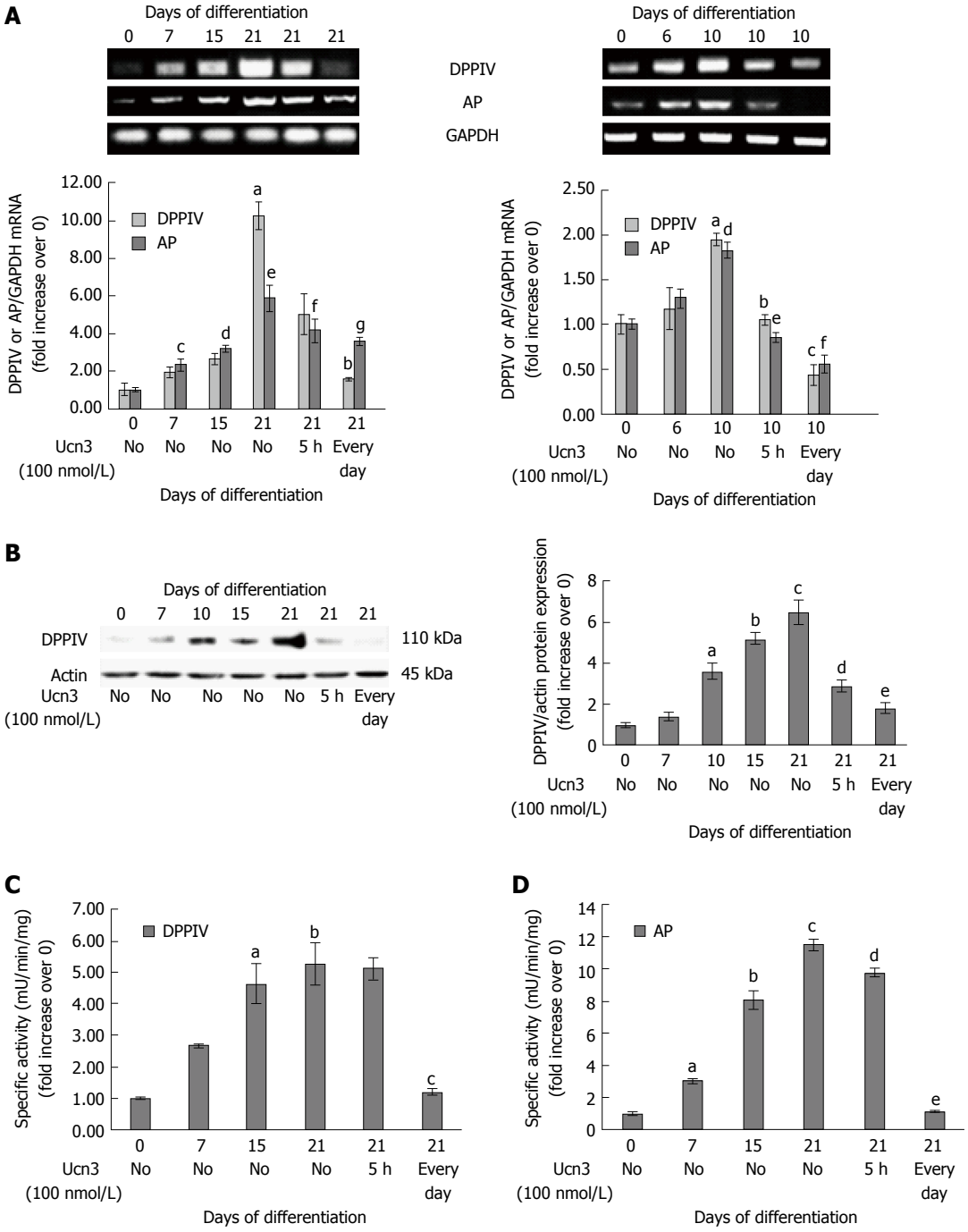

Figure 6 Corticotropin releasing factor receptor 2 signaling alters expression of characteristic markers of enterocyte differentiation. A: Right panel: Detection of DPPIV and AP mRNA expression by RT-PCR during the kinetic of Caco-2 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 21 d differentiated cells. GAPDH served as a housekeeping control. Quantification of KLF4 and AP mRNA from RT-PCR assays (lower panel). Data were expressed as fold increase of KLF4 or AP/GAPDH mRNA levels of differentiated (D7, D15, D21) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. $^{a,c}P < 0.01$ *vs* undifferentiated Caco-2 cells (D0), $^{d,e}P < 0.001$ *vs* D0, $^{b}P < 0.05$ *vs* differentiated Caco-2 cells (D21), $^{f}P < 0.01$ *vs* D21, $^{g}P < 0.001$ *vs* D21; Note that normality of distribution was not respected for DPPIV PCR. Left panel: Detection of DPPIV and AP mRNA expression by RT-PCR during the kinetic of HT-29 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 10 d differentiated cells. GAPDH served as a housekeeping control. Quantification of DPPIV and AP mRNA from RT-PCR assays (lower panel). Data were expressed as fold increase of DPPIV and AP/GAPDH mRNA levels of differentiated (D6 and D10) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. Data represents means of three different experiments ± SEM. $^{a,d}P < 0.01$ *vs* undifferentiated HT-29 cells (D0); $^{b}P < 0.01$ *vs* early differentiated HT-29 cells (D10), $^{c,e,f}P < 0.001$ *vs* D10; B: Left panel: Detection of DPPIV protein expression by western blot during the kinetic of Caco-2 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 21 d differentiated cells. Actin served as a loading control. Right panel: Quantification of DPPIV protein levels from western blot analysis. Data were expressed as fold increase of DPPIV/actin protein levels of differentiated (D7, D15, D21) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. $^{a}P < 0.01$ *vs* undifferentiated Caco-2 cells (D0), $^{b,c}P < 0.001$ *vs* D0, $^{d}P < 0.01$ *vs* differentiated Caco-2 cells (D21), $^{e}P < 0.001$ *vs* D21. C and D: Detection of DPPIV (C) and AP (D) enzymatic activities during the kinetic of Caco-2 cell differentiation and after acute (5 h) or chronic (every day) exposure to 100 nmol/L Ucn3 of 21 d differentiated cells. Data were expressed as fold increase of enzymatic activities of differentiated (D7, D15, D21) *vs* undifferentiated cells (D0). Data represents means of three different experiments ± SEM. For C: $^{a,b}P < 0.001$ *vs* undifferentiated Caco-2 cells (D0); $^{c}P < 0.01$ *vs* differentiated Caco-2 cells (D21). For D: $^{a}P < 0.05$ *vs* undifferentiated Caco-2 cells (D0), $^{b,c}P < 0.001$ *vs* D0, $^{d}P < 0.05$ *vs* differentiated Caco-2 cells (D21) and $^{e}P < 0.001$ *vs* D21.

crypts in the colon[59]. However, the CRF2 receptor localization remains unclear. The receptor may have either apical[59] or basolateral location[60]. Our confocal microscopy experiments, carried out on tissue sections but also on carcinoma cell lines, are in favor of a distribution of CRF2 at the basal membrane of IEC in the colon of Sprague Dawley rats and at the baso-lateral membrane of early differentiated HT-29 cells (data not shown) supporting the hypothesis of a localization of CRF2 oriented to the mucosa. Such localization of CRF2 could allow: (1) paracrine stimulation by the ENS or by ligands produced by the immune cells; and (2) autocrine stimulation by ligands produced by IEC themselves.

The role of the components of the CRF system in cancer is correlated with their level of expression and also with their cellular distribution. Indeed, CRF components are expressed and secreted by various cancer cells but the cytoplasmic distribution of CRF2 observed in de-differentiated or metastatic tumors was associated with diseases at a more advanced stage[61-63]. Accordingly, our results describe aberrant expression of CRF2 and ligands in both CRC tumors and cell lines, depending on their grade and/or differentiation status suggesting a role for CRF2 in the loss of cellular organization and tumor progression[25].

Role of CRF2 signaling in epithelial permeability

Stress causes disruption of intestinal epithelial barrier homeostasis, leading to worsening of GI disorders such as IBD and irritable bowel syndrome (IBS)[18,64,65]. Changes in intestinal mucosa permeability have been attributed to an alteration of junctional molecules, whose expression is affected by the actively inflamed status in IBD or IBS patients, in particular the expression of ZO-1, occludin, E-cadherin and desmoglein-2[66]. To understand the role of the CRFergic system in the regulation of intestinal homeostasis, approaches have been developed based either on the inhibition of ligands or the inhibition of receptors, *via* genetic or pharmacological extinction or *via* administration of peripheral CRF or various CRF antagonists[19,67-72]. Stress-induced modulation of colonic permeability seems to be either CRF1- or CRF2- dependent. This modulation has been attributed to eosinophils or ENS-derived CRF which activate mast cells that in turn induce TNFα and protease release as well as finally disruption of TJ[73-75]. Therefore, very few studies have investigated the activation of CRF2 in IEC, whose expression is increased under inflammatory conditions in patients with IBD[60,76] or under stressful conditions (personal data). Our results show that the increase in intestinal permeability induced by Ucn3 is due to CRF2 signaling since the effect was abolished by a pre-treatment with Astressin 2B, a CRF2 antagonist. The increase in both para- and trans-cellular permeabilities is correlated with an alteration of intercellular adhesion complexes such as AJ and TJ in more differentiated cells. Indeed, CRF2 signaling modifies the membrane distribution of AJ and TJ proteins. According to the increase of both E-cadherin and p120ctn in LR of HT-29 cells during their early differentiation (from day 0 to 10) our data are consistent with the previously described role of LR in intercellular complex maturation[6,7]. Treatment of these cells with Ucn3 (2 h) induced a decrease of E-cadherin and p120ctn in LR. These changes coincide with the decrease in TEER observed in differentiated HT-29 cells after 2 h of treatment with Ucn3, suggesting that the disorganization of AJ following activation of CRF2 could be responsible for an increase in intestinal permeability. Such alterations in the distribution of proteins of intercellular junctions are found in inflammatory models. Indeed, the presence of TJ proteins is decreased in LR of IEC of rats subjected to TNBS-induced colitis[77]. The stimulation of CRF2 could promote the activation of Src, a kinase that is strongly involved in the regulation of AJ[25]. Src kinase allows insertion of AJ by phosphorylation of PI3K[78]. Conversely, if AJ are already in place, phosphorylation of Src leads to AJ destabilization[79] by phosphorylation of p120ctn[80], leading to endocytosis of E-Cadherin which will then be ubiquitinylated and degraded by the proteasome[81]. These elements are consistent with the disappearance of p120ctn and E-cadherin from LR under Ucn3 treatment (2 h). At 5 h of treatment with Ucn3, the expression profile of E-cadherin and p120ctn in the different fractions of the gradient is intermediate between that of the undifferentiated cells (D0) *vs* the differentiated cells (D10). We suppose that there is a membrane enrichment of E-cadherin that could result from more active recycling, restoring the AJ. Furthermore, the increase in the expression of E-cadherin and p120ctn in fractions 5-6 and 7-8 could illustrate newly formed junctions whose maturation state is intermediate between untreated HT-29 cells at D0 and D10. These results are also consistent with the partial return of TEER to the level of HT-29 cells treated with Ucn3.

The phenomena of pinocytosis and autophagy involved in the internalization of the extracellular apical medium, forming the vacuoles, could correspond to the refringent zone observed in phase contrast microscopy. These results are consistent with the increase in HRP flux observed in different stress models in animals[69,82,83]. The appearance of intracellular vacuoles in the monolayer of differentiated HT-29 cells treated with Ucn3 could also contribute to the increase in permeability. Furthermore, the persistence of these structures after 5 h of treatment with Ucn3 while E-cadherin molecules accumulate in LR suggests that independent mechanisms could contribute to the increase in permeability. A similar phenotype was observed in presence of forskolin, an activator of adenylate cyclase (data not shown). This suggests that AMPc production is involved in this mechanism. CRF2

activation in IEC could also lead to an AMPc-mediated increase in trans-cellular permeability.

Regulation of enterocytic differentiation by CRF2

Gland atrophy and mucin depletion have been observed during chronic colitis[84,85]. Knowing the protective function of mucins in the epithelial barrier, it seems likely that in response to the inflammation induced by crypt epithelial damage and ulceration, the epithelium responds by increasing proliferation and thus, reducing differentiation[86]. Estienne *et al*[34] showed that activation of CRF1 and CRF2 induced by MD markedly induce alterations in the differentiation of IEC resulting in a hyperplasia of enteroendocrine cells and depletion of Paneth and Goblet cells, which may lead to the development of an epithelial barrier defect. The decrease does not exceed the duration of the cell population renewal of the epithelium suggesting that in order to induce a long-term effect, CRF signaling must affect stem cells. Analyzing various characteristic markers of IEC differentiation, we demonstrate that CRF2 signaling could also affect enterocyte-like differentiation of human adenocarcinoma cell lines. AJ-mediated signaling is linked to activation of Wnt, PI3K/Akt and FGF pathways that are particularly important in intestinal cell proliferation and differentiation[87-89]. Recurrent alteration of AJ may decrease the activation of the signaling pathways necessary for the progression of enterocyte differentiation. Indeed, chronic administration of Ucn3 during differentiation delays the increase in DPPIV and PA activity found in differentiated Caco-2 cells. Regulation of DPPIV activity is correlated with a down-regulation of DPPIV protein expression following Ucn3 exposure. As it might be expected, the exposure to chronic Ucn3 compared to a single exposure (acute stress) has more severe consequences on enzyme activities. *In vivo,* the alteration generated by an acute stress does not exceed 5 d or the time of the cellular renewal of the intestinal epithelium. In these experiments, the colonic epithelial barrier is morphologically altered, the expression of mRNAs coding for the TJ proteins is reduced and the differentiation of the colon cells is modified[68]. The use of chronic stress (5-10 d of repeated exposure to stressors) is thought to reflect more accurately the daily stressors of humans. Indeed, the exposure to chronic water avoidance stress (WAS) leads to enhanced ultrastructural abnormalities in the epithelium, characterized by reduced crypt length (caused by increased apoptosis) and increased cell proliferation, in an attempt to replace damaged cells and decrease cell differentiation. The presence of undifferentiated cells within the epithelium may be responsible for the lackiness of the epithelial barrier[90].

Another way by which Ucn3 could affect enterocyte differentiation is by modulating ECM proteins. Indeed, we found that exposure of HT-29 cells to Ucn3 induced remodeling of ECM components by regulating both metalloprotease secretion and laminin 332 deposit ([25] and unpublished data). The role of ECM in the reinforcement of E-cadherin-dependent signaling has been previously described in Caco-2 cells[91]. Activation of integrins, which occurs after Caco-2 cells are cultured on complex ECM stimulates the expression of apolipoprotein A IV, a marker of IEC differentiation. Similarly, laminin 111 isoform induces the expression of the sucrase-isomaltase in Caco-2 cells, together with the nuclear shuttling of nucleolin, a transcriptional regulator[92]. This process requires the activation of the p38/MAPK signaling pathway, a cascade known to activate the Caudal type homeobox 2 (Cdx2) protein, a key intestinal transcription factor involved in intestinal epithelial differentiation[93]. In contrast, laminin 332 expression is more associated with inflammation processes such as the restitution of inflamed epithelium but also tumor invasion[94].

KLF4 is a zinc finger transcription factor enriched in the intestinal epithelium[95]. Based on *in situ* and immunohistochemical experiments, KLF4 localizes to the upper region of the colonic crypt and the villi of small intestine, its expression increases during differentiation along the crypt-villus axis and during intestinal cell maturation *in vitro* of either the absorptive or the goblet cell lineage[54]. Furthermore, KLF4 is down-regulated in CRC and has been proposed as a tumor suppressor[53,56,73,96-99]. KLF4 exhibits an expression pattern similar to APC, a negative regulator of the Wnt pathway[100]. We confirmed an increased expression of KLF4 in colonic tumor epithelial cells during their differentiation at both transcriptional and post-transcriptional levels. However, we found that CRF2 activation by Ucn3 decreases the mRNA and protein expression of KLF4 in differentiated HT-29 and Caco-2 cells, suggesting that stress may regulate intestinal homeostasis by controlling transcription factor expression. Along these lines, it has been demonstrated that WAS reduces goblet cell number and mucin 2 synthesis *via* decreased Cdx2 expression[101]. Since KLF4 expression is dependent of Cdx2 in human colon cancer cells, our findings are consistent with these reports[73]. KLF4 regulates both differentiation and growth which is likely fundamental for maintenance of intestinal homeostasis and for its tumor suppressor activity[102]. In this regard, KLF4 transcriptional targets are involved in cell differentiation such as genes coding for laminin 111, AP and villin[103,104]. The Ucn3-mediated down-regulation of KLF4 in differentiated Caco-2 cells may result in the decrease of DPPIV and AP activities. The mechanism by which CRF2 activation regulates intestinal homeostasis remains unknown. Many observations are in favor of an indirect effect of CRF2 action on KLF4 expression: (1) KLF4 expression increases during the process of cell differentiation whereas CRF2 expression decreases; (2) KLF4 expression is transcriptionally regulated during cell differentiation in both cell lines;

and (3) KLF4 expression is increased with the establishment of mature intercellular junctions. One possible mechanism is that by dissociating intercellular junctions Ucn3-mediated activation of CRF2 signaling could indirectly regulate KLF4 expression at both transcriptional and post-transcriptional levels. Indeed, we have found that CRF2 signaling induces an alteration of AJ, a process associated with the delocalization of AJ proteins. Release of β-ctn from AJ complexes leads to the transcriptional activity of β-ctn/Tcf signaling which plays a crucial role in homeostasis and transformation of the intestinal mucosa[10,105]. Furthermore, it has been proposed that elevated β-ctn/Tcf signaling reduces levels of KLF4[54]. We observed that Ucn3-mediated cell dissociation is associated with nuclear translocation of β-ctn (data not shown). The decrease in expression of KLF4 following activation of CRF2 could therefore induce: (1) an increase in proliferation; (2) an altered intestinal epithelial differentiation; (3) a loss of mucus cells causing a large decrease in mucus and thus leading to mechanical (by chyme) and chemical (by digestive juices) changes in the epithelium; (4) an impairment of the release of defenses promoting bacterial proliferation; and (5) an epithelio-mesenchymal transition at the origin of tumor development.

In conclusion, we showed that CRF2 signaling induces alterations in both the epithelium permeability and the differentiation of colonic carcinoma cell lines. To our knowledge, this is the first report showing that CRF2 signaling modifies the enterocyte-like differentiation process. On one hand, by altering the differentiation of enterocyte cells, stress could lead to the development of epithelial barrier defects and alterations of mucosal function, contributing to the enhancement of GI disorders. On the other hand, by altering the differentiation status of cancer cells, stress may contribute to tumor development. CRF2 could therefore play a role in tumor progression by loss of cellular contacts, increased cell permeability and decreased KLF4 expression.

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COMMENTS

Background

In the last decade, the influence of stress (psychological and environmental) on pathogenesis received increased awareness. This is particularly true for gastrointestinal (GI) disorders occurring in inflammatory bowel disease (IBD) and colorectal cancers (CRC). In human as well as in animal models, stress has been described to alter intestinal barrier integrity and function. Furthermore stress neuromediators, such as urocortins (Ucn 2/3) and their receptor the Corticotropin Releasing Factor 2 (CRF2) were upregulated in poorly differentiated CRC and promote metastatic potential through an epithelial-mesenchymal transition-like process. These observations led us to investigate the role of CRF2 signaling in the modulation of epithelial permeability and enterocyte-like cell differentiation.

Research frontiers

Patients with IBD often suffer from intestinal inflammatory flares that favor the development of colitis associated cancer. Stress could favor the development and/or aggravation of GI disorders by inducing flares. However the mechanisms involved in this process are still poorly understood, but are mainly associated with epithelial barrier dysfunction.

Innovations and breakthroughs

The authors' results reinforce the role of stress in the development and/or aggravation of GI disorders. While stress has been described to modulate the fate of secretory epithelial cells, its role on enterocyte differentiation remains unknown. New findings from our work indicate that: (1) CRF2 protein is preferentially expressed in undifferentiated epithelial cells from the crypts of colon and in human cell lines; (2) Ucn3-mediated CRF2 signaling alters enterocyte differentiation by down-regulating KLF4 transcription factor expression; (3) this effect relies on alterations of cell permeability and cellular adhesion junctions; and (4) the effect on cell differentiation is greater following chronic exposure to Ucn3 rather than acute exposure. The impact of stress on enterocyte differentiation may contribute to barrier dysfunction and development of GI disorders.

Applications

To our knowledge, this is the first report showing that CRF2 signaling modifies the enterocyte-like differentiation process. On one hand, by altering the differentiation of enterocyte cells, stress could lead to the development of epithelial barrier defects and alterations of mucosal function, contributing to the enhancement of GI disorders. On the other hand, stress-induced loss of cellular differentiation favors tumor initiation and progression. Thus a better understanding of the underlying mechanisms associated with stress will propose new therapeutic targets.

Terminology

The CRFergic system is a central element of the stress response constituted of specific stress neuromediators, such as corticotropin releasing factor or its analogs urocortins (Ucn 1, 2 and 3) and their receptors CRF1 and CRF2.

Peer-review

This manuscript demonstrated that Ucn3-induced CRF2 signaling could modulate intestinal epithelial cell differentiation and epithelial cell permeability. The authors found CRF2 was associated with a poor differentiated status of IEC. Then, they proved CRF2 signaling altered the trans- and para-cellular permeability, and delayed colonic cell differentiation. In general, the work would be potentially useful to reveal the roles of CRF2 signaling in tumor progression.

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ORIGINAL ARTICLE

Basic Study

Bone marrow-derived monocyte infusion improves hepatic fibrosis by decreasing osteopontin, TGF-β1, IL-13 and oxidative stress

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Abstract

AIM

To evaluate the therapeutic effects of bone marrow-derived $CD11b^+CD14^+$ monocytes in a murine model of chronic liver damage.

METHODS

Chronic liver damage was induced in C57BL/6 mice by administration of carbon tetrachloride and ethanol for 6 mo. Bone marrow-derived monocytes isolated by immunomagnetic separation were used for therapy. The cell transplantation effects were evaluated by morphometry, biochemical assessment, immunohistochemistry and enzyme-linked immunosorbent assay.

RESULTS

$CD11b^+CD14^+$ monocyte therapy significantly reduced liver fibrosis and increased hepatic glutathione levels. Levels of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL)-6 and IL-1β, in addition to pro-fibrotic factors, such as IL-13, transforming growth factor-β1 and tissue inhibitor of metalloproteinase-1 also decreased, while IL-10 and matrix metalloproteinase-9 increased in the monocyte-treated group. $CD11b^+CD14^+$ monocyte transplantation caused significant changes in the hepatic expression of α-smooth muscle actin and osteopontin.

CONCLUSION

Monocyte therapy is capable of bringing about improvement of liver fibrosis by reducing oxidative stress and inflammation, as well as increasing anti-fibrogenic factors.

Key words: Monocytes; Bone marrow mononuclear cells; Cell therapy; Macrophages; Glutathione; Liver fibrosis

Core tip: Chronic inflammation is now recognized as a central player in the development of liver fibrosis. Studies have shown that activated macrophages establish a link between chronic inflammation and fibrosis in various organs. The present study evaluated the therapeutic effects of bone marrow-derived $CD11b^+CD14^+$ monocytes in a murine model of liver damage. The results show that mice with transplants had improvement of liver fibrosis by way of a reduction in oxidative stress and inflammation and an increase in anti-fibrogenic factors. The study demonstrates the beneficial effects of cellular therapy in liver fibrosis and also reports on the important modulatory mechanisms involved.

de Souza VCA, Pereira TA, Teixeira VW, Carvalho H, de Castro MCAB, D'assunção CG, de Barros AF, Carvalho CL, de Lorena VMB, Costa VMA, Teixeira AAC, Figueiredo RCBQ, de Oliveira SA. Bone marrow-derived monocyte infusion improves hepatic fibrosis by decreasing osteopontin, TGF-β1, IL-13 and oxidative stress. *World J Gastroenterol* 2017; 23(28): 5146-5157 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5146.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5146

INTRODUCTION

Abuse of alcohol, infections caused by hepatitis viruses B and C, and nonalcoholic steatohepatitis (NASH) are the main causes of liver tissue damage[1]. These risk factors can lead to focal or diffuse hepatocellular degeneration and necrosis. Persistent inflammatory stimulus in the liver can induce the formation of fibrous tissue, and ultimately lead to the development of liver cirrhosis[2]. Hepatic stellate cells (HSCs) play an important role in liver fibrogenesis because they are the main source of secreted extracellular matrix (ECM) components[3]. When severe liver damage occurs, HSCs are activated, mainly by the action of transforming growth factor-beta (TGF-β), tumor necrosis factor-alpha (TNF-α) and reactive oxygen species (ROS) produced by damaged hepatocytes or liver-resident macrophages[4].

The ECM components comprise various types of proteins, including osteopontin (OPN)[5], a pro-inflammatory cytokine that modulates the pro-fibrogenic phenotype of HSCs and is involved in many physiological and pathological processes, including inflammation, fibrosis and angiogenesis[5,6]. OPN has also been described as a mediator induced by the Hedgehog pathway and plays an important role in the repair of acute and chronic liver damage, both in humans and experimental models[7,8].

The remodeling of fibrous tissue is a complex mechanism by which multiple cell types, capable of producing molecules such as matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), play an important role in the synthesis and degradation of the ECM[9]. In chronic liver damage, the establishment of hepatic fibrosis is directly related to MMP/TIMP imbalance[10], thereby showing that MMPs and TIMPs may be potent therapeutic targets[11].

Although important advances in the knowledge of

chronic liver diseases have been made, the existing treatments are still limited. New, more effective and less invasive therapeutic strategies are therefore needed. In this context, several studies of regenerative medicine have demonstrated the potential of cell therapy as a promising emerging treatment for liver diseases[12] and various cell populations have been investigated to this end[12,13]. Bone marrow mononuclear cells (BMMCs) have shown promising results in both experimental[14] and clinical[15,16] studies. Previous studies of experimental models of liver injury have demonstrated that cell therapy is able to decrease mortality[17] and levels of hepatic fibrosis[14], improve biochemical parameters[18], increase MMP-9 expression[19], and reduce levels of TGF-β1[20] and galectin-3 expression[14].

Identifying which components of the BMMC population are responsible for the beneficial effects of cell therapy is extremely important for clinical application. Recent studies have reported that monocytes may have important therapeutic potential in chronic liver diseases[21,22]. These cells are the precursors of the heterogeneous macrophage population involved in liver repair responses. In the liver, macrophages perform various functions, such as phagocytosis and cytokine production, which are important in the inflammatory response to damage, liver fibrosis and degradation of ECM[23,24]. *In vitro* assays have shown that monocytes maintained in culture supplemented with hepatocyte growth factor exhibited similar behavior to those hepatic cells obtained from the liver culture[21]. One preclinical study has shown that cellular therapy with cultured macrophages decreases murine liver fibrosis and this is followed by changes in the levels of some mediators involved in liver repair[22].

Although these findings are of great importance, information about the functions of monocyte/macrophage cell lineages in cell therapy for liver diseases is still limited. The present study evaluated the therapeutic potential of bone marrow-derived monocytes in a murine model of chronic liver damage induced by carbon tetrachloride (CCl_4) and ethanol.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (4-6 wk of age), weighing 20-23 g were obtained from the Animal Breeding Center Laboratory Fundação Oswaldo Cruz (FIOCRUZ, Rio de Janeiro, Rio de Janeiro, Brazil), and housed in the animal research facility in the Aggeu Magalhães Research Center (CPqAM; FIOCRUZ, Recife, Pernambuco, Brazil). The animal protocol was designed to minimize pain or discomfort to the animals, which were maintained in rooms with a controlled temperature (22 ± 2 ℃) and humidity (55% ± 10%) environment under continuous air renovation conditions. Animals were housed in a 12-h light/12-h dark cycle and free access to food (Nuvilab, Curitiba, Paraná, Brazil) and water. Experimental procedures were in accordance with the ethical standards of the Oswaldo Cruz Foundation and approved by the Ethics Committee for the Certified Use of Animals (CEUA-CPqAM 15/2011).

Chronic liver damage and experimental design

Chronic liver damage was induced in the mice by orogastric administration of 200 μL of 20% CCl_4 solution diluted in olive oil, in twice weekly doses[14]. The mice also received a 5% ethanol solution in water *ad libitum*. CCl_4 treatment was carried out for 6 mo. The mice were randomly divided into four experimental groups with chronic hepatic damage: Group Ⅰ: Control mice (normal mice) (n = 5); Group Ⅱ: Saline-treated mice (n = 5); Group Ⅲ: Mice treated with BMMCs (n = 5); Group Ⅳ: Mice treated with BMMC-derived monocytes (n = 5).

Isolation of BMMCs and monocytes

Bone marrow was harvested from the femurs and tibiae of donor C57BL/6 mice (n = 15) and BMMCs were purified by centrifugation in a Ficoll gradient (Histopaque 1119 and 1077; Sigma Aldrich, St Louis, MO, United States) at 1000 × g for 15 min. This protocol facilitates the rapid recovery of viable BMMCs using two ready-to-use separation mediums in conjunction. The BMMC preparation was used to isolate monocytes by way of the immunomagnetic cell separation system. For this, the BMMCs (approximately 10^7 cells/mL) were incubated with anti-CD11b antibodies conjugated to magnetic microbeads (MACS units; Miltenyi Biotec™, Bergisch Gladbach, Germany), washed and passed through a magnetic column (MACS; Miltenyi Biotec™), where $CD11b^+$ monocytes were retained and recovered in a buffer [0.5% PBS/0.5% bovine serum albumin (BSA) + 2 mmol/L EDTA]. Finally, the cells were washed and re-suspended in 0.9% sterile saline, which was later infused into the mice.

Cell characterization

The BMMCs and monocytes obtained by immunomagnetic separation were first incubated with Anti-CD11b (PE Rat Anti-Mouse CD11b, M1/70 clone, BD Pharmingen™, San Jose, CA, United States), Anti-CD14 (FITC Rat Anti-Mouse CD14, rmC5-3 clone; BD Pharmingen™), Anti-CD45 (APC Rat Anti-Mouse CD45, 30-F11 clone; BD Pharmingen™), Anti-CD34 (PE Rat Anti-Mouse CD34, RAM34 clone; BD Pharmingen™) and Anti-Ly6A (FITC Rat Anti-Mouse Ly-6A/E, D& clone; BD Pharmingen™). After 30 min of incubation, cells were washed with 2 mL of PBS wash solution (PBS with 0.5% BSA + 0.1% sodium azide), centrifuged at 400 × g for 5 min and then resuspended in 300 μL of the PBS wash solution.

The samples were then phenotypically characterized by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, United States). A minimum of 10000 events/sample were collected. The cell population

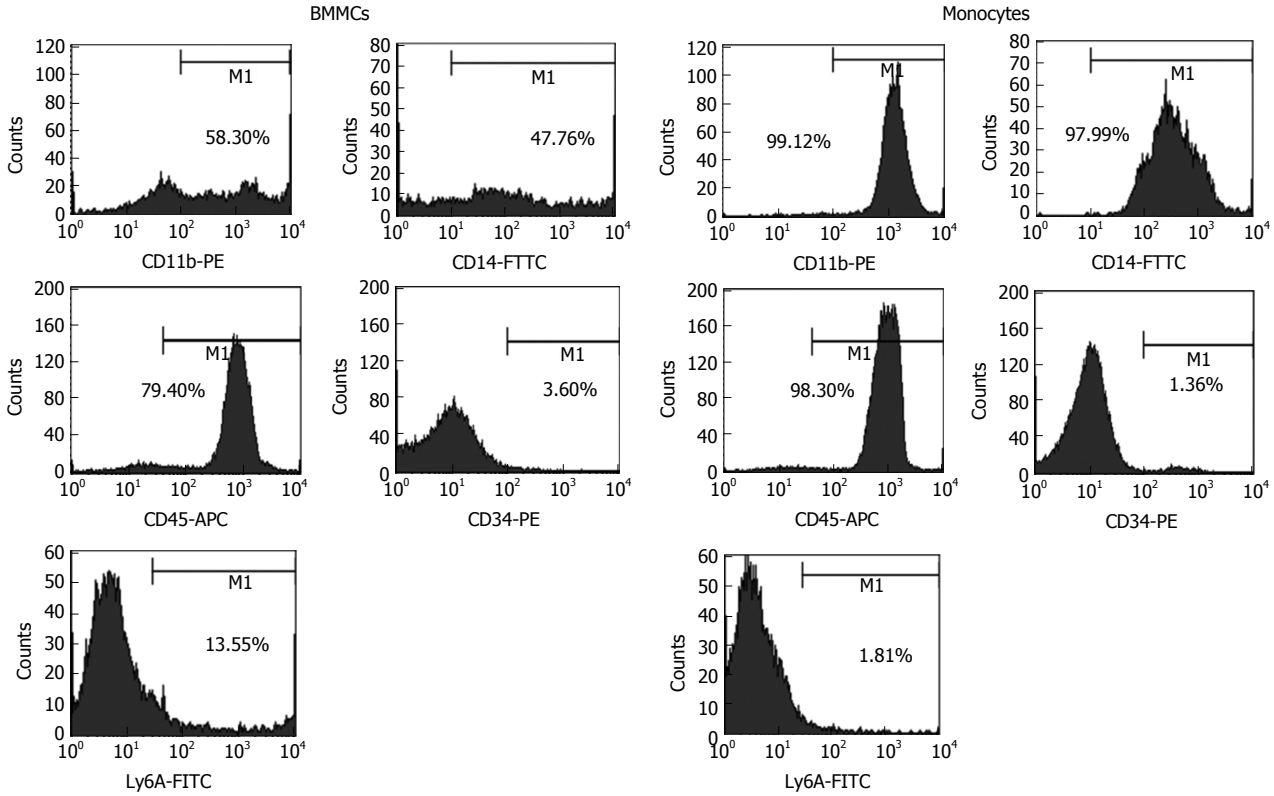

Figure 1 Representative FACS histograms of bone marrow mononuclear cells and CD11b$^+$ monocytes isolated by immunomagnetic separation. BMMCs: Bone marrow mononuclear cells.

obtained by immunomagnetic separation presented the following phenotype distribution: 99.12% CD11b$^+$; 97.99% CD14$^+$; 98.3% CD45$^+$; 1.36% CD34$^+$; and 1.81% Ly6A$^+$ cells; differing from those of BMMCs, which were: 58.3% CD11b$^+$; 47.76% CD14$^+$; 79.4% CD45$^+$; 3.6% CD34$^+$; and 13.55% Ly6A$^+$ cells. These distinctive profiles demonstrated enrichment of homogeneous monocytes population in our cell preparation. Figure 1 shows representative FACS histograms of BMMCs and CD11b$^+$ monocytes isolated by immunomagnetic separation.

Cell infusion in mice with chronic liver damage

At 6 mo after treatment with CCl_4/ethanol, bone marrow-derived CD11b$^+$CD14$^+$ monocytes and BMMCs were administered endovenously to the mice (10^6 cells/animal) for 3 consecutive wk. At 2 mo after transplantation, mice were euthanized and the liver and the spleen were extracted for further analysis (Figure 2).

Morphometric evaluation

In order to characterize and quantify liver fibrosis, treated and non-treated samples were fixed for 24 h in 10% formalin, embedded in paraffin, sectioned (5 μm) and stained with picro-Sirius. Images were obtained using an optical microscope (DM LB 2; Leica Microsystems, Wetzlar, Germany) equipped with a JVC TK (model - C 1380; Leica, Allendale, NJ, United States) and analyzed using the Image Analysis Processing System Leica QWIN, version 2.6 MC (Leica, Cambridge, United Kingdom). Ten microscopic fields (100 × magnification) containing fibrous tissue areas were chosen for quantification. To detect and quantify Kupffer cells, the histological sections were stained with hematoxylin and eosin (H&E) and observed under an optical microscope (DM LB 2; Leica Microsystems). The cell counts were performed in 10 fields/sections (400 × magnification).

Hydroxyproline (Hyp) assay

Liver samples (approximately 200 mg) were immersed in 6N HCl at approximately 120 ℃ for 18 h, followed by filtration. The hydroxyproline (Hyp) concentration was determined by a colorimetric assay at 558 nm as previously described[25] and expressed as nmol/g liver.

Immunohistochemistry analysis

Immunohistochemistry was carried out to evaluate the activated HSCs (alpha-smooth muscle actin, α-SMA) and OPN. To stain α-SMA, liver sections (5 μm) were initially deparaffinized with xylene, dehydrated in increasing concentrations of ethanol, incubated overnight with biotinylated antibody anti-α-SMA (Santa Cruz Biotechnology, Dallas, TX, United States), and then incubated with streptavidin-peroxidase for 10 min. For OPN staining, the samples were incubated overnight with primary anti-OPN antibodies (AF808; R&D Systems, Minneapolis, MN, United States), as previously described[5]. Thereafter, a secondary

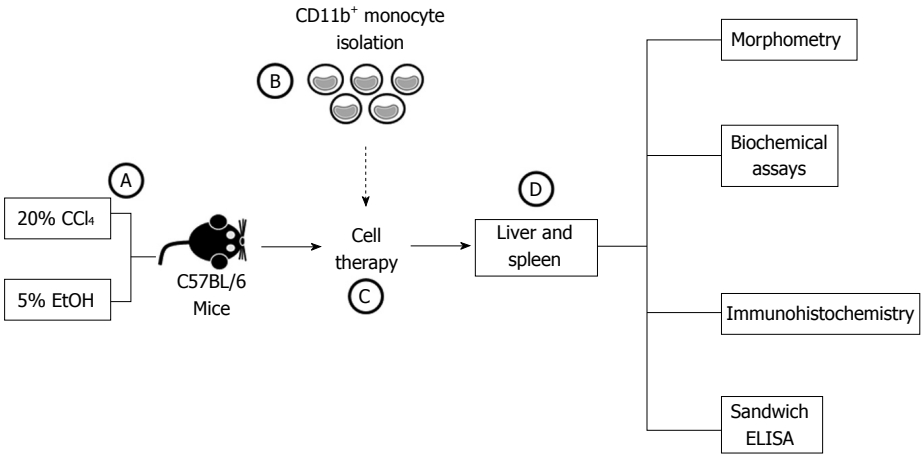

Figure 2 Schematic flowchart of experimental design. A: Male C57BL/6 mice underwent chronic administration of CCl_4 and EtOH solutions for 6 mo; B: Bone marrow mononuclear cells were harvested from C57BL/6 donor mice for $CD11b^+$ monocyte isolation using immunomagnetic separation; C: Chronically liver-damaged mice underwent cell therapy; D: After 2 mo, effects of the treatment were evaluated using morphometric, biochemical, immunohistochemistry and sandwich ELISA analysis.

antibody bound to a synthetic polymer conjugate with peroxidase (horseradish peroxidase, HRP). 3,3' diaminobenzidine was used for staining. The sections were counterstained with Harris hematoxylin. The staining was measured in 10-fields/sections (200 × magnification) using the Image Analysis Processing System Leica QWIN, version 2.6 MC.

Glutathione measurement

To evaluate oxidative stress, the amount of glutathione (GSH) was quantified using liver fragments from mice submitted to the cell therapy and those that were not. The liver fragments were weighed, macerated in 5% metaphosphoric acid solution and centrifuged at 12000 × *g* at 4 ℃ for 10 min. GSH was detected using the Glutathione Assay Kit (Sigma Aldrich) and measured with a microplate reader at 415 nm (BioRad, Hercules, CA, United States).

Enzyme-linked immunosorbent assay

Frozen liver fragments (approximately 100 mg) were homogenized in a lysis buffer (50 mmol/L Tris-HCl, 300 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.02% sodium azide) containing a protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were centrifuged at 16000 × *g* for 15 min at 4 ℃ and supernatants were used to quantify the levels of TNF-α, IL-6, IL-1β, IL-13, IL-10**,** IL-17, IL-23, TGF-β1, MMP-9 and TIMP-1 by way of a sandwich enzyme-linked immunosorbent assay assay following the manufacturers' instructions (IL-13, IL-17, IL-23, MMP-9 and TIMP-1 by R&D Systems; TGF-β1: Human/Mouse TGF-beta1 by e-Bioscience, San Diego, CA, United States; TNF-α, IL-1β, IL-6 and IL-10 using the OptEIA Set for Mouse by BD Biosciences). Samples were read at a 450 nm wavelength using a microplate reader (Model 3550; Thermo Fisher Scientific, Waltham, MA, United States). The concentration of TGF-β1 was also determined from supernatants of splenocyte culture obtained from mice used in the study, as previously described[26]. The cytokine concentration was expressed in pg/mL.

Statistical analysis

Quantitative data were submitted to the normality test (Shapiro-Wilk's). Differences were evaluated using the ANOVA test for parametric analysis, and the Kruskal-Wallis test with post-hoc Dunn's test for non-parametric analysis. Statistical analyses were performed using Prism Software (version 5.0; GraphPad Software, San Diego, CA, United States) and Bioestat 5.3 (Mamirauá Institute, Manaus, AM, Brazil). A P value of < 0.05 was considered statistically significant. Data were expressed as mean values (mean ± SEM).

RESULTS

Monocyte therapy alters hepatic fibrosis

Morphometric analysis, 2 mo after therapy, showed a significant decrease in fibrotic areas in the liver from $CD11b^+CD14^+$ in the monocytes-treated group compared to the saline-treated group ($P < 0.001$; Figure 3B, D and E). This decrease was also found in mice treated with BMMCs ($P < 0.05$; Figure 3B, C and E). A marked reduction in the amount of Hyp was also observed in the group that received monocyte treatment ($P < 0.01$; Figure 3F). The number of Kupffer cells significantly increased in the monocyte-treated ($P < 0.001$) and BMMC-treated ($P < 0.01$) groups, when compared to the saline-treated group (Figure 3G).

To test whether $CD11b^+CD14^+$ monocyte transplantation was able to alter the number of activated HSCs, α-SMA-positive cells were assessed by immunohistochemistry. As shown in Figure 4, α-SMA-positive cells in the hepatic parenchyma were decreased in the mice that received monocytes ($P < 0.01$; Figure 4D and E) as well as in the BMMC-treated group ($P < 0.05$;

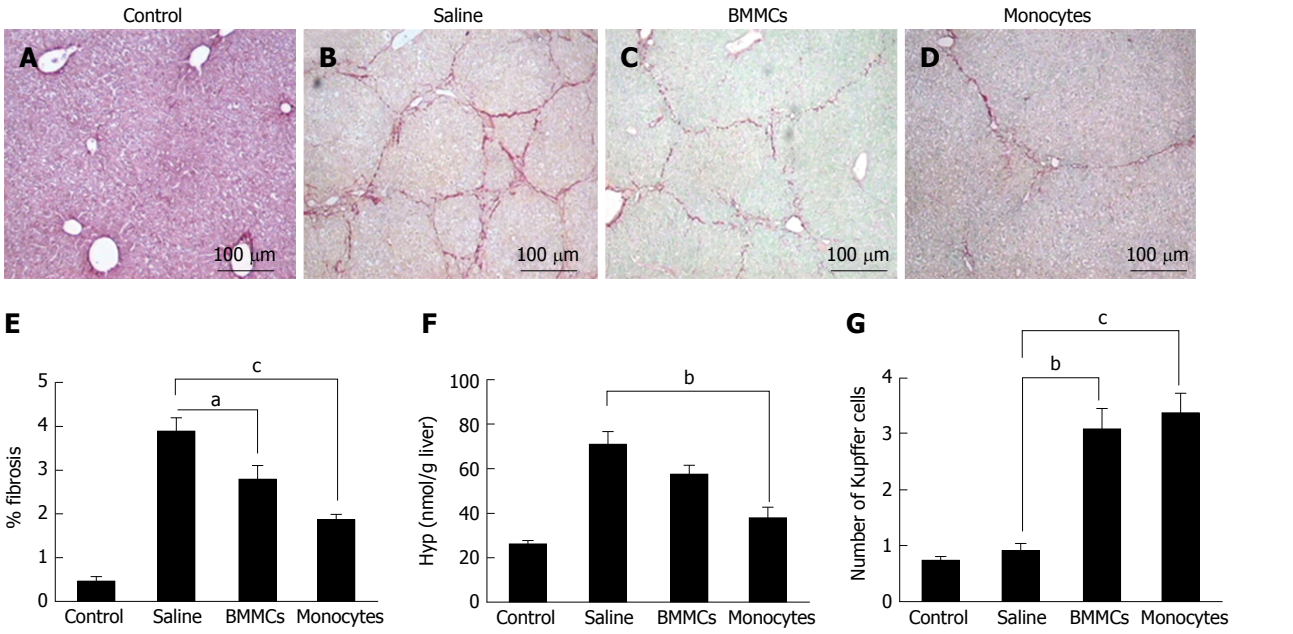

Figure 3 Photomicrographs of histological liver sections stained with picro-Sirius red. A-D: The figure shows hepatic collagens in (A) control mice, mice after CCl_4 administration and treatment with (B) saline, (C) BMMCs and (D) BMMC-derived monocytes (picro-Sirius red; magnification × 100); E: Morphometric evaluation of picro-Sirius Red-stained sections; F: Hydroxyproline in liver fragments of mice undergoing cell transplantation; G: Kupffer cell count in hematoxylin-eosin-stained histological liver sections from mice who underwent $CD11b^+CD14^+$ monocyte therapy and BMMC-treated mice. $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.001$. BMMCs: Bone marrow mononuclear cells.

Figure 4C and E) compared with the group treated with saline (Figure 4B and E). Furthermore, OPN also decreased after $CD11b^+CD14^+$ monocyte therapy (Figure 5).

Monocyte transplantation reduces hepatic inflammatory and pro-fibrotic cytokine levels

To investigate the mechanisms involved in the improvement of hepatic fibrosis after $CD11b^+CD14^+$ monocyte therapy, the levels of hepatic inflammatory and pro-fibrotic cytokine were quantified. The levels of TNF-α, IL-1β and IL-6 in liver lysates were significantly lower in the $CD11b^+CD14^+$ monocytes-treated group ($P < 0.05$; Figure 6A-C). IL-13 (Figure 6D) and TGF-β1 (Figure 7A), important fibrogenic mediators, were significantly lower compared to those in mice treated with saline ($P < 0.05$). In the supernatant splenocyte culture obtained from the monocyte- and BMMC-treated groups, there was a significant decrease in TGF-β1 compared with the saline-treated mice ($P < 0.05$; Figure 7B). IL-17 cytokine levels were also lower in animals undergoing cell transplantation ($P < 0.01$; Figure 7C). A trend was also observed for decreased IL-23 cytokine levels (Figure 7D).

Monocyte therapy altered MMP-9, TIMP-1 and IL-10 hepatic levels

The levels of MMP-9 and TIMP-1, two relevant factors associated with liver fibrosis, were evaluated. A significant increase in the production of MMP-9 was found in animals treated with $CD11b^+CD14^+$ monocytes and BMMCs ($P < 0.05$; Figure 8A). Interestingly, TIMP-1 levels were significantly lower in $CD11b^+CD14^+$ monocyte-treated mice ($P < 0.05$; Figure 8B). The monocyte-treated group also showed significantly increased levels of IL-10 in comparison with the saline-treated group ($P < 0.05$; Figure 8C).

Monocyte therapy increases GSH levels

GSH levels were determined to evaluate the influence of $CD11b^+CD14^+$ monocyte therapy on oxidative stress. Monocyte-treated mice with chronic liver damage had significantly higher levels of this antioxidant molecule than the saline-treated group ($P < 0.05$; Figure 8D).

DISCUSSION

The present study corroborates the importance of monocytes/macrophages in liver repair. These may act to regulate some significant fibrogenic pathways, in a murine model of chronic liver damage. Monocytes/macrophages are cells with great plasticity and, depending on the tissue microenvironment, may be caused to adopt a profile that contributes to resolution/regression of experimental hepatic fibrosis[24].

The results of the present study demonstrate that transplantation of BMMC-derived $CD11b^+CD14^+$ monocytes had beneficial effects on liver lesions, thereby causing a significant reduction in fibrosis, mainly by regulating important cytokines involved in the liver repair process. Previous work carried out by our group has already shown a decrease in collagen levels in a liver undergoing BMMC therapy[14]. However, the results obtained in the present study demonstrated an improvement in these parameters on BMMC-derived $CD11b^+CD14^+$ monocyte infusion, with an almost

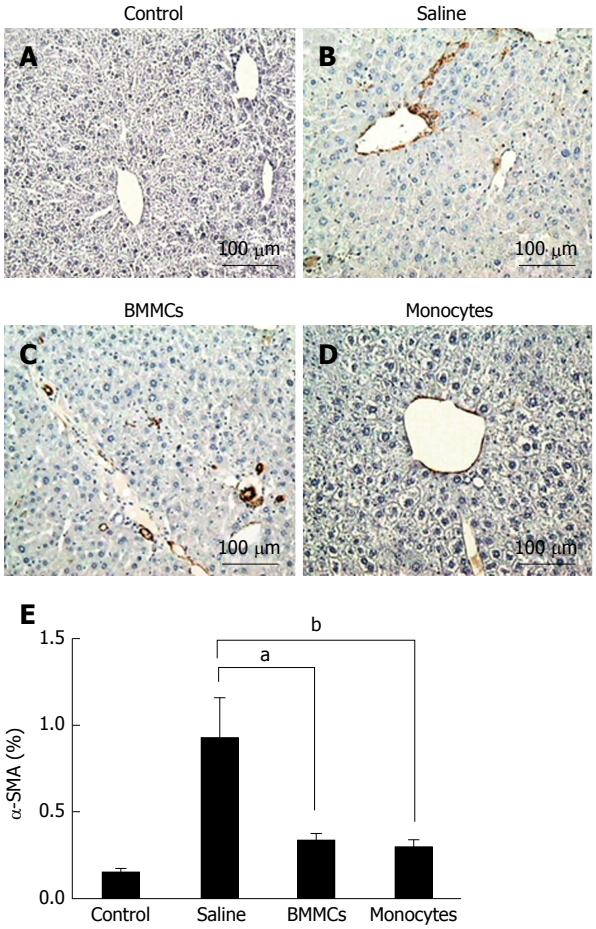

Figure 4 Immunohistochemistry for detection of α-SMA$^+$ hepatic stellate cells in histological sections. A-D: Control (A), saline-treated (B), BMMCs-treated (C) and CD11b$^+$CD14$^+$ monocyte-treated (D) groups of mice (magnification × 200); E: Measurement of α-SMA$^+$ hepatic stellate cells at 2 mo after treatment with CD11b$^+$CD14$^+$ monocytes and BMMCs. $^aP < 0.05$; $^bP < 0.01$. BMMCs: Bone marrow mononuclear cells.

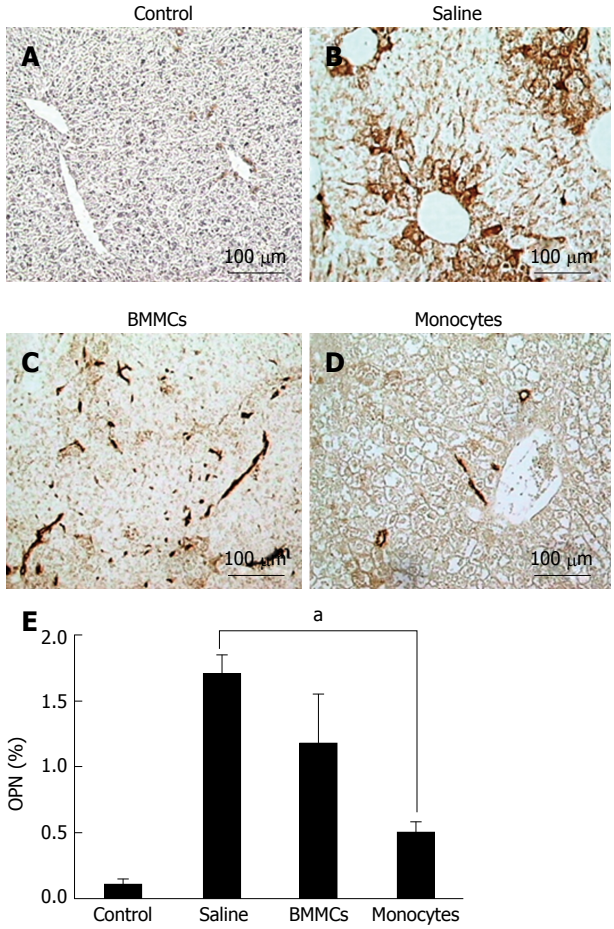

Figure 5 Immunohistochemistry for detection of osteopontin in histological sections. A-D: Control (A), saline-treated (B), BMMCs-treated (C) and CD11b$^+$CD14$^+$ monocyte-treated (D) groups of mice (magnification × 200); E: Levels of hepatic OPN at 2 mo after treatment with CD11b$^+$CD14$^+$ monocytes. $^aP < 0.05$. OPN: Osteopontin. BMMCs: Bone marrow mononuclear cells.

2-fold decrease in the collagen levels using the same experimental model.

Macrophages, important mediators of inflammatory responses, have a dichotomous response when activated, assuming a classical (M1) or alternative (M2) pathways phenotype depending on the environmental stimulus[27]. The increase in the number of hepatic resident macrophages (Kupffer cells) after cell therapy observed in our study suggests that the subsets of restorative macrophages are involved in the tissue repair by inhibiting the production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6)[28]. Previous studies have reported the role of macrophages in mediating liver fibrogenesis, and have proposed using macrophage subpopulations during liver damage and repair[23,29]. Treatments carried out in experimental models have shown that the infusion of bone marrow-derived macrophages decreases fibrous tissue, and enhances hepatic regeneration[22,30,31].

The decrease in fibrous liver tissue observed in the present study may be associated with the lower number of activated HSCs found. The pro-fibrogenic role of this cell type has been already reported in the literature, indicating a direct relationship between murine liver fibrosis and the rise in the number of activated HSCs[3,4]. In this regard, some studies have reported a decrease in the number of α-SMA$^+$ cells in murine models of liver damage treated with BMMCs. This decrease is probably due to an alteration in the modulation of HSCs by specific cytokines and growth factors, including TGF-β1, TNF-α and ROS, produced by hepatocytes in a damaged liver[32]. As activation of HSCs is mediated by autocrine and paracrine signaling and these cells not only secrete cytokines but also respond to them[32], it was hypothesized that BMMC-derived CD11b$^+$CD14$^+$ monocytes modulate the activity of HSCs by regulating the secretion of cytokines and growth factors.

Production of the pro-inflammatory cytokine profiles of TNF-α, IL-1β and IL-6 were inhibited in mice submitted to liver damage and treated with CD11b$^+$CD14$^+$ monocytes. Furthermore, there was an increase in the synthesis of IL-10 cytokine, which is known for its T helper (Th)2 profile and anti-

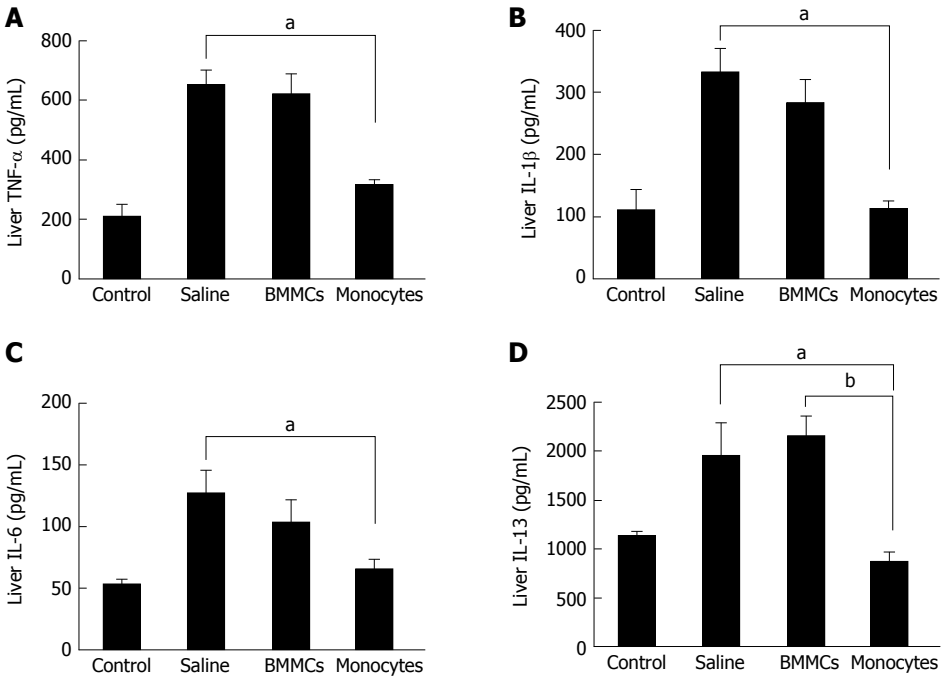

Figure 6 Effects of monocyte therapy on the cytokine profile of tumor necrosis factor-alpha (A), interleukin-1β (B), interleukin-6 (C) and interleukin-13 (D), as measured by enzyme-linked immunosorbent assay. Data are represented graphically as the mean ± SEM of 5 mice/group. [a]*P* < 0.05; [b]*P* < 0.01. BMMCs: Bone marrow mononuclear cells; IL: Interleukin; TNF-α: Tumor necrosis factor-alpha.

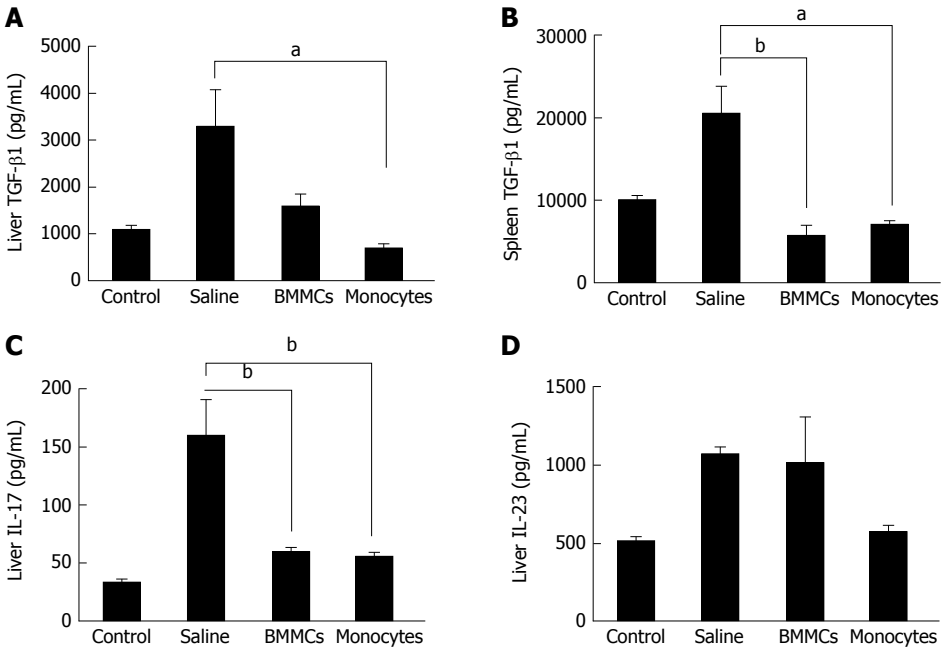

Figure 7 Effects of monocyte-based therapy on chronically liver-damaged mice. A: Hepatic levels of TGF-β1; B: Splenic levels of TGF-β1; C and D: Hepatic levels of IL-17 (C) and IL-23 (D). [a]*P* < 0.05; [b]*P* < 0.01. TGF-β: Transforming growth factor-beta; IL: Interleukin; BMMCs: Bone marrow mononuclear cells.

inflammatory activity[33]. These results show the influence of $CD11b^{+}CD14^{+}$ monocyte infusion in the hepatic production of inflammation and fibrogenesis mediators. The modulation of inflammation during liver repair processes by way of increased expression of IL-10 and inhibition of the production of TNF-α, IL-1β and IL-6 is well described in the literature[34]. Because of their role in activating and proliferating HSCs, these cytokines have been implicated in the pathogenesis of chronic liver inflammation, mainly by increasing the production of collagen and regulating MMPs and TIMPs in liver damage[35,36]. Gene therapy studies have shown that the overexpression of IL-10 reduces the expression of pro-fibrotic molecules such as TGF-β1 and TNF-α[36], thereby down-regulating the inflammatory response and reducing activated HSCs, which ultimately leads to

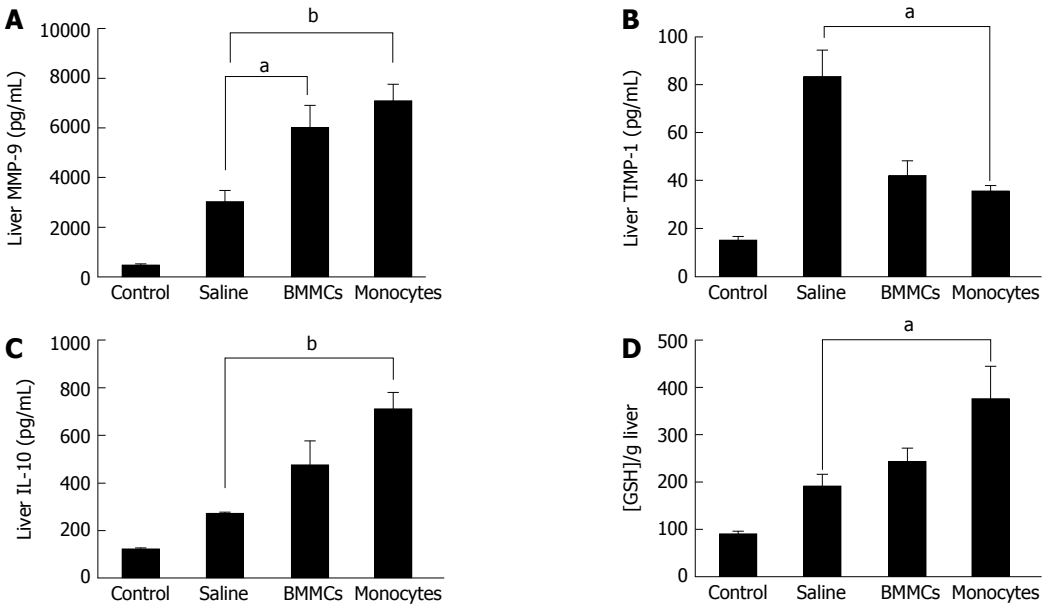

Figure 8 Effects of monocyte-based therapy on hepatic levels of matrix metalloproteinases-9 (A), tissue inhibitors of metalloproteinase-1 (B), interleukin-10 (C) and glutathione (D), in chronically liver-damaged mice. $^{a}P < 0.05$; $^{b}P < 0.01$. MMP-9: Matrix metalloproteinases-9; TIMP: Tissue inhibitors of metalloproteinase; IL: Interleukin; GSH: Glutathione; BMMCs: Bone marrow mononuclear cells.

the reestablishment of liver function[35,36].

The present study found a significant decrease in TGF-β1 levels in both the extracts of liver protein and the supernatant of cultured splenocytes. These results corroborate other findings of the study, thereby indicating that transplanted monocytes play an important anti-fibrogenic role. TGF-β1 is a growth factor which plays a crucial role in initiating and maintaining liver fibrogenesis[4]. This factor is directly involved in activating HSCs and synthesizing ECM components, mostly in type Ⅰ collagen[4]. It also plays an important role in inhibiting the degradation of ECM, stimulating the decrease of MMP synthesis and increasing the production of TIMPs, which leads to excessive deposition of collagen and the establishment of hepatic fibrosis[2]. Previous studies have associated the improvement of experimental liver fibrosis after BMMC-treatment with the reduction in TGF-β1 levels[20,22]. The results of the present study suggest that monocyte therapy acts through this fibrogenic pathway, thereby contributing to reducing liver fibrosis in mice.

The present investigation showed that cell transplantation caused a significant decrease in IL-17 levels, an effector pro-inflammatory cytokine, produced by $CD4^+$ T cells[37]. This mediator induces the recruitment of inflammatory factors into liver cells and also directly activates natural hepatic immunity systems, such as those mediated by neutrophils and dendritic cells, to release cytokines that perpetuate chronic inflammation[38]. Previous reports have reported that Th17 cells are able to participate in the pathogenesis of hepatic lesions associated with hepatitis B virus[39]. Recently, emerging evidence has indicated that IL-17 may be implicated in the induction of liver fibrosis, contributing to the activation of HSCs *in vitro*[39].

OPN is a glycoprotein expressed in a variety of tissues, mainly found in ECM and sites of healing wounds[40]. Studies have shown that this protein is highly expressed in fibrotic liver tissue and influences the function of hepatic progenitors[41]. Under this condition, increases in the level of TGF-β and activation of HSCs could be also observed[6,41]. It thus seems reasonable to suppose that deactivation of OPN could lead to attenuation of liver fibrosis[1,8]. The results of the present study accordingly showed a significant decrease in the production of OPN and in the number of activated HSCs.

GSH is an important antioxidant molecule that acts as a modulator of redox signaling, cell proliferation, apoptosis, immune responses and fibrogenesis[42,43]. Reduced levels of this molecule have been found in preclinical fibrosis models and in human fibrotic diseases[42]. A previous study has shown that higher GSH production inhibits the fibrogenic activity of TGF-β1[43]. The present study also found an increase in this molecule after $CD11b^+CD14^+$ monocyte transplantation, suggesting an association between the anti-fibrotic effects observed in the monocyte-treated group and increased antioxidant activity of this cell population.

Alterations in the quantities of some molecules involved in fibrogenesis, as well as fibrous tissue remodeling, were assayed in this study. The $CD11b^+CD14^+$ monocyte therapy in mice with chronic liver damage caused an increase in MMP-9 hepatic levels. Previous studies have associated reduced liver fibrosis with fibrous tissue degradation[3]. MMP-9 plays an important role in resolving liver fibrosis and has been considered a potent therapeutic target[11]. Yang *et al*[44] suggest

that, in the hepatic microenvironment, macrophage subpopulations play an anti-fibrotic role, as they express several MMPs, including MMP-9, which are directly involved in degrading ECM, facilitating the resolution of hepatic fibrosis.

$CD11b^{+}CD14^{+}$ monocyte transplantation gave rise to a reduction in hepatic TIMP-1 and IL-13, two important pro-fibrogenic mediators. TIMPs are involved in the regulation of fibrogenic response by inhibiting the enzymatic activity of MMPs, having an anti-apoptotic effect on HSCs[9]. The presence of high quantities of these inhibitors in chronically damaged hepatic tissue may contribute to the establishment of liver fibrosis[45]. IL-13 is a cytokine associated with severe forms of schistosomal liver fibrosis as well as non-schistosomiasis liver diseases[46]. IL-13 is considered one of the central mediators in liver pathogenesis and is involved in TGF-β1 production by liver cells, besides its ability to induce progenitor cells to transdifferentiate into myofibroblasts, which produce collagen[47]. The data produced by the present study corroborates the protective role of monocytes/macrophages in tissue repair processes, by way of fibrogenic pathways.

Several studies have attempted to identify and to correlate different macrophage profiles to tissue repair processes[29,30,48]. Ramachandran *et al*[29] found that $Ly6C^{low}$ macrophages secrete large amounts of fibrolytic MMPs such as MMP-9 and MMP-13, as well as IL-10. Therefore, the increase in secretion of MMP-9 and IL-10 observed in this study suggests a down-regulation of the activation pathways that lead to the chronic inflammatory response.

In conclusion, the present study shows the important contribution of bone marrow-derived monocyte/macrophage cell therapy for improving the state of liver fibrosis in a murine model of chronic liver damage. These cells act to modulate inflammation and fibrogenesis and regulate the oxidative stress caused by damaged tissue. Further studies should be conducted to establish a promising therapeutic tool for treating chronic liver diseases.

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COMMENTS

Background

Chronic liver disease is characterized by alterations in the process of tissue repair, such as the excessive deposition of fibrous tissue and the inhibition of the dynamics of regeneration. The knowledge on bone marrow cell therapy has opened new perspectives towards treatment of hepatic diseases. However, the cell types involved in liver recovery have not been fully elucidated. Monocytes have emerged as one set of potential candidates, due to their plasticity and involvement in inflammation and tissue repair.

Research frontiers

Previous experiments have already shown that bone marrow cell transplantation promotes improvement in the experimental model of liver fibrosis. The monocyte/macrophage lineage may have important therapeutic potential in chronic liver diseases.

Innovations and breakthroughs

This is an innovative study that evaluated the effects of monocyte transplantation isolated from bone marrow mononuclear cells, by morphological, biochemical and immunological assays.

Applications

Experimental hepatic fibrosis improvement after cell therapy reinforces the potential involvement of monocytes/macrophages in liver repair, being able to acquire pro-resolute profile, acting in the regulation of some relevant inflammatory and fibrogenic pathways.

Terminology

Bone marrow mononuclear cells are used to collectively denominate bone marrow cells, whose nuclei are unilobulated and which lack granules in the cytoplasm. This cell population includes hematopoietic progenitor cells, lymphoid cells (lymphocytes, plasma cells) and monocytes.

Peer-review

The authors addressed an interesting, clinically relevant and important issue aiming to modify the state of chronic liver disease. To approach this goal, the authors purified bone marrow-derived $CD11b^{high}$ monocytes, which were transfused to mice prior to the administration of the provoking agents, *i.e.*, ethanol and carbon tetrachloride. Using a C57BL/6 mouse model system, they showed that the transfusion of monocytes was more effective to decrease IL-13 levels in the liver as compared to the infusion of BMMCs. The authors also demonstrated that monocyte transfusion could reduce the size of the fibrotic area, the amount of hydroxyproline and the concentration of pro-inflammatory cytokines, while the levels of interleukin-10 cytokine and the number of Kupffer cells in the liver were increased as compared to saline transfusion. Due to the limited information of cell-based therapies in chronic inflammatory diseases, the identification of immunostimulatory and regulatory pathways in a preclinical setting and in the context of liver metabolism is of high importance.

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ORIGINAL ARTICLE

Basic Study

Single amino acid mutation of SR-BI decreases infectivity of hepatitis C virus derived from cell culture in a cell culture model

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Abstract

AIM

To investigate the effect of a single amino acid mutation in human class B scavenger receptor I (SR-BI) on the infectivity of cell culture-derived hepatitis C virus (HCVcc) in SR-BI knock-down Huh7-siSR-BI cells.

METHODS

Site-directed mutagenesis was used to construct the SR-BI S112F mutation, and the mutation was confirmed by nucleotide sequencing. SR-BI knock-down Huh7-siSR-BI cells were transfected with SR-BI S112F, SR-BI wild type (WT) and control plasmids, and then infected with HCVpp (HCV pseudoparticles) and hepatitis C virus derived from cell culture (HCVcc). A fluorescence assay was performed to analyze the effect of the S112F mutation on HCV entry; quantitative real-time PCR, immunofluorescence, and Western blot assays were used to analyze the effect of the S112F mutation on HCV infectivity. CHO cells expressing WT and SR-BI S112F were incubated with the HCV E2 protein expressed in HEK 293T cells, and flow cytometry was performed to examine the ability of SR-BI S112F to bind to the HCV E2 protein. Huh7-siSR-BI cells were transfected with SR-BI WT and the S112F mutant, and

then DiI-HDL was added and images captured under the microscope to assess the ability of SR-BI S112F to take up HDL.

RESULTS

The SR-BI S112F mutation was successfully constructed. The S112F mutation decreased the expression of the SR-BI mRNA and protein. SR-BI S112F decreased HCV entry and HCVcc infectivity in Huh7-siSR-BI cells. The S112F mutation impaired the binding of SR-BI to HCV E2 protein and decreased the HDL uptake of SR-BI.

CONCLUSION

The S112F single amino acid mutation in SR-BI decreased the levels of the SR-BI mRNA and protein, as well as the ability of SR-BI to bind to the HCV E2 protein. Amino acid 112 in SR-BI plays important roles in HCV entry and the infectivity of HCVcc *in vitro*.

Key words: Hepatitis C virus; Class B scavenger receptor I; Mutant; Infectivity

Core tip: Human class B scavenger receptor I (SR-BI) plays important roles in both host lipid metabolism and the entry of hepatitis C virus (HCV). Single nucleotide polymorphisms (SNPs) in the host genome that affect the virus-host interaction have received increasing attention in recent years. Several SR-BI SNPs have been reported to affect the high-density lipoprotein cholesterol levels in populations carrying SR-BI mutations; however, the impact of SR-BI SNPs on HCV infection has not been studied intensively. Based on our results, the S112F single amino acid mutation in SR-BI inhibited the infectivity of hepatitis C virus derived from cell culture in a cell culture model by downregulating the expression of the SR-BI protein.

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. The HCV genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins[1]. HCV infection is a global health problem, with an estimated 180 million persons infected worldwide, and HCV infection is the leading cause of cirrhosis and hepatocellular carcinoma[2]. The prevalence of chronic HCV infection in China was 3.2% in 1992 and 0.4% in 2006. Recent reports from the Chinese Ministry of Health have identified 70861 cases in 2006 and 201622 cases in 2012[3]. The most recent investigation showed a prevalence of HCV infection of 3.0% in northeastern China[4]. In recent years, the Chinese government has increased its investment in the prevention and control of viral hepatitis. However, an effective vaccine is not available and treatment with the combination of interferon and ribavirin therapy produces a response in approximately half of infected patients. More recently, a new therapy comprising novel direct-acting antivirals (DAAs), such as protease inhibitors (telaprevir, boceprevir and simeprevir) and an RNA polymerase inhibitor (sofosbuvir), increased the sustained virological response rate in HCV-infected patients[5-7]. However, the DAA therapy also produces significant side effects[8]. Therefore, novel anti-HCV methods, including host targets, are still needed.

HCV entry is a multi-step process that requires many host molecules, including the tetraspanin molecule CD81, human class B scavenger receptor I (SR-BI), and the tight-junction proteins claudin-1 (CLDN1) and occludin (OCLN)[9-12]. Among these proteins, SR-BI plays a crucial role since both SR-BI and its ligand lipoprotein are involved in the HCV entry process[13,14]. SR-BI has a number of common ligands, including high-density lipoprotein (HDL), low-density lipoprotein (LDL) and oxidized LDL[13]. As shown in a study by Dreux *et al*[15], HDL enhances the infectivity of HCVpp (HCV pseudoparticles) and hepatitis C virus derived from cell culture (HCVcc).

SR-BI was originally defined as a class B scavenger receptor in a family that includes CD36, LIMP II (lysosome membrane protein II), and SR-BII (a form of SR-BI with an alternate C-terminal cytoplasmic tail)[16]. SR-BI is a lipoprotein receptor composed of 509 amino acids (aa) in which the cytoplasmic C- and N-terminal domains are separated by a large extracellular domain. As an HDL receptor, SR-BI mediates selective uptake of HDL-derived cholesteryl ester (CE) *in vitro* and *in vivo*[17]. The conformation of the extracellular domain is important for the binding of SR-BI and HDL, and hence affects the function of SR-BI[18]. Eleven N-linked glycosylation sites (aa102, 108, 116, 173, 212, 227, 255, 288, 310, 330 and 383) have been identified in the extracellular domain, and two glycosylation sites (Asn108 and Asn173) were proven to be indispensable for the expression and function of SR-BI[19]. In addition to mediating selective CE transport, SR-BI has been shown to play important roles in many human diseases, including atherosclerosis, apoptosis, immune responses, HCV and dengue virus entry, and malaria parasite infection[20].

In recent years, the influence of single nucleotide polymorphisms (SNPs) in the host genome on the virus-host interaction has received increasing attention. For HCV, most studies have focused on SNPs

in the *IL28B* gene and HCV prognosis[21]. Currently, few reports on virus entry and host genomic SNPs have been published[22]. For SR-BI, researchers have focused on its regulation of HDL-cholesterol and other metabolites, and a very recent report showed that polymorphisms in the SR-BI gene are associated with the virological response in HCV-infected patients[23,24]. According to the results of a GWAS (genome-wide association study) of 10000 individuals, SNPs in the SR-BI gene are associated with a small, but significant elevation in plasma HDL-cholesterol levels[25]. Recently, a single loss-of-function mutation (P297S) in SR-BI was identified, and the mutation increased HDL-cholesterol levels and reduced cholesterol efflux from macrophages[26]. Subsequently, two novel missense mutations, S112F (nucleotide C588T) and T175A (nucleotide A776T) were also shown to be associated with elevated HDL-cholesterol levels[27,28]. However, few studies have investigated how the mutations impact HCV infection and development. In this study, we studied the effects of the SR-BI S112F single amino acid mutation on the infectivity of HCVcc using a cell culture model.

MATERIALS AND METHODS

Materials

The Huh7 cell line, human embryonic kidney (HEK) 293T cell line and Huh7-siSR-BI cell line are maintained in our laboratory. Briefly, SR-BI shRNA was designed, cloned into the pGP-Lenti3 vector (Biovector, Science Lab, Beijing), and the positive recombinant Lv-SR-BI-shRNA vector was verified. This vector and helper plasmids were co-transfected into HEK 293T cells. The recombinant lenti-SR-BI-shRNA virus was used to infect Huh7 cells. Puromycin was added for screening, and real-time PCR and Western blot were conducted to detect the levels of the SR-BI mRNA and protein, respectively; finally, the Huh7-siSR-BI cell line was obtained[29].

Cells were grown in complete Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL, United States), 1 mmol/L -glutamine, 100 nmol/L nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Construction of SR-BI S112F plasmid expressing the SR-BI single amino acid mutation

The SR-BI S112F mutation was introduced into pcDNA-SR-BI (maintained in our lab) using a Quick Change Lightning Site-Directed Mutagenesis Kit (Stratagene, CA, United States), according to the manufacturer's instructions, with the primers (S112F-F: 5'-ACAAC GACACCGTG*T*TCTTCCTCGAGTACCGCACCT-3' and S112F-R: 5'-AGGTGCGGTACTCGAGG*A*AGAACA CGGTGTCGTTGT-3'; the italic letters represent the mutant nucleotide). The presence of the desired mutation was confirmed using nucleotide sequencing (Invitrogen, CA, United States).

Quantitative real-time RT-PCR (qRT-PCR)

RNA was isolated from harvested cells using TRIzol reagent (Invitrogen), and RNA was prepared according to the manufacturer's instructions. RNA obtained from 1 × 10^5 cell equivalents was analyzed using RT-PCR. RNA samples were transcribed into cDNA using random primers and then quantitatively analyzed with the specific primers SR-BI-F: 5'-GCTGCAGGAA GCAAAACTGT-3' and SR-BI-R: 5'-CCAGTAGAAAAGGG TCACAGG-3' using the quantitative RT-PCR kit (Applied Biosystems, United States). Genome copy numbers were normalized to GAPDH levels determined in parallel (GAPDH-F: 5'-TGACTTCAACAGCGACACCCA-3'; GAPDH-R: 5'-CACCCTGTTGCTGTAGCCAAA-3') using the comparative cycle threshold values.

Immunofluorescence assays

Huh7 and SR-BI knock-down Huh7-siSR-BI cells were cultured in collagen-coated 96-well plates at a density of 1 × 10^4 cells/well on the day before transfection. Cells were transfected with the SR-BI wild type (WT) and SR-BI S112F plasmids using Lipofectamine™ (Invitrogen, CA, United States), according to the manufacturer's instructions. Fresh cell medium was replaced 6 h after transfection and the cells were cultured for 48 h. Cells were washed with PBS, fixed with cold methanol, and then stained with an anti-SR-BI mouse monoclonal antibody (mAb) (1:1000 dilution, BD, United States) or serum from HCV-infected patients (1:100 dilution) for 2 h at room temperature (RT). After being washed with PBS, cells were reacted with an Alexa Fluor 488-conjugated anti-mouse antibody or human IgG antibodies (1:1000 dilution, BD, United States). Nuclei were stained with DAPI (1:2000 dilution, BD, United States). Images were captured and infected foci were counted under a fluorescence microscope (Olympus IX81, Japan).

Western blot analysis

Protein extracts from transfected cells were prepared in a modified RIPA buffer containing 0.5% SDS and a protease inhibitor cocktail (Complete mini; Roche) on ice. After centrifugation, protein concentrations were determined using the BCA method (Beyotime). The proteins were separated using 10% (w/v) SDS-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA, United States) using a Trans-Blot apparatus (Bio-Rad). Membranes were blocked with 5% nonfat milk, incubated with a primary anti-SR-BI mouse mAb (1:2000 dilution, BD), and detected using a horseradish peroxidase (HRP)-conjugated species-specific secondary antibody (Santa Cruz Biotechnology). Immunoreactivity was visualized using enhanced chemiluminescence (GE Healthcare).

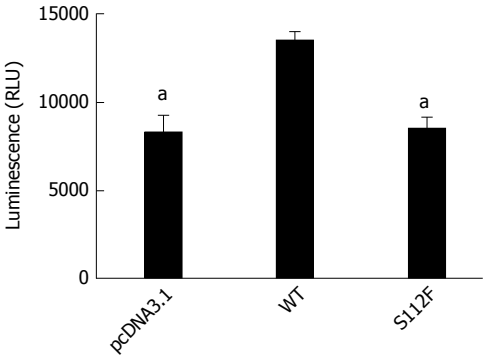

Figure 1 Effect of the class B scavenger receptor I single amino acid mutant on hepatitis C virus entry. Huh7-siSR-BI cells were seeded on a 96-well plate, cultured overnight, and transfected with pcDNA3.1 (NC), pcDNA-SR-BI, or pcDNA-SR-BI/S112F. Three days after transfection, HCVpp were added to the cell culture. Cells were harvested 72 h post infection and cell lysates were analyzed using a fluorescence assay. The data are presented as the mean ± SEM from three independent experiments ($^aP < 0.05$). WT: Wild type.

HCVcc production and HCV infection assay

The pJFH1 plasmid, which was kindly provided by Dr. Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan), was used as template to generate HCVcc as described[30]. Briefly, pJFH-1 was linearized to generate the template used in the *in vitro* transcription reaction to produce the viral RNA using the MEGAscript kit (Promega, Madison, WI, United States). Huh7 cells were then transfected with JFH-1 transcripts by electroporation. Cell culture supernatants were collected 5 d later and the viral titer was quantified. For the infection assay, Huh7-siSR-BI cells were seeded in a 96-well plate, cultured in 10% FBS-DMEM overnight, and then infected with HCVcc. The cells were cultured for 48 h before the infectivity was measured using the immunofluorescence assays.

HCVpp production and HCV entry assay

HCVpp were generated as described[31]. Briefly, HEK 293T cells were transfected with plasmids encoding the HCV envelope proteins, Gag/Pol (pLP1) and Rev (pLP2) and the pLenti6 transfer vector (Invitrogen) expressing the luciferase gene. Vesicular stomatitis virus pseudoparticles were produced as controls. Cell culture supernatants were collected 2 d later and the viral titer was quantified using immunofluorescence assay (IFA). For the HCV entry assay, Huh7-siSR-BI cells were seeded on a 96-well plate, cultured overnight, and transfected with pCDNA-SR-BI WT and mutant plasmids, inoculated with HCVpp, and then cultured for 72 h before the entry of HCVpp was measured using a fluorescence assay.

SR-BI and HCV E2 protein binding assay

The SR-BI and HCV E2 protein binding assay was performed using a FACS-based assay, as previously described[32]. Briefly, 4×10^5 CHO cells expressing SR-BI were incubated with equivalent amounts of HCV E2 protein (transient expression in HEK 293T cells) for 1 h at RT, washed twice with PBS, and incubated with an anti-E2 mAb (1 h at RT). After labeling with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), the mean fluorescence intensity was quantified by flow cytometry (Beckman Coulter, Fullerton, CA, United States).

DiI-HDL uptake assay of SR-BI WT and mutant

Huh7-siSR-BI cells were seeded in a 96-well plate at a density of 2×10^4 cells/well and cultured for 24 h. Cells were transfected with the SR-BI WT and mutant S112F plasmids using Lipofectamine™. Complete culture medium was replaced 6 h after transfection and the cells cultured for 48 h before DiI-HDL (200 μg/mL, Alfa Aesar, United States) was added and the cells were cultured for an additional 4 h. Nuclei were stained with Hoechst (Merk, NJ, United States) for 10 min, and cells were observed and images captured using a microscope.

Statistical analysis

The error bars represent the SD of means from at least three independent experiments. Statistical significance was analyzed using Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Generation of the SR-BI S112F mutant

The pcDNA-SR-BI/S112F vector expressing the SR-BI single amino acid mutant was obtained by site-directed mutagenesis and confirmed by nucleotide sequencing. The Ser at site 112 was replaced with Phe in the mutant.

SR-BI S112F mutation decreases HCV entry

Huh7-siSR-BI cells were transfected with pcDNA3.1, pcDNA-SR-BI, or pcDNA-SR-BI/S112F first, and infected with HCVpp expressing the luciferase gene 3 d after transfection. Fluorescence was detected 3 d later. Compared with cells transfected with the pcDNA3.1 vector control and pcDNA-SR-BI WT control, SR-BI S112F decreased the entry of HCV in Huh7-siSR-BI cells (Figure 1).

SR-BI S112F mutation decreases HCVcc infection

Huh7-siSR-BI cells were seeded in 96-well plates (for IFA) or 24-well plates (for qRT-PCR) and transfected with pcDNA3.1, pcDNA-SR-BI, or pcDNA-SR-BI/S112F; HCVcc (10^3FFU/mL) was added 24 h after transfection. Cells were harvested 72 h after infection and RNA was isolated for qRT-PCR. IFA was performed 48 h later. According to the IFA results, the expression of the HCV protein was decreased in the pcDNA-SR-BI/S112F group (Figure 2A). Based on the qRT-PCR results, the level of the HCV RNA was decreased in the pcDNA-SR-BI/S112F group (0.711 of the WT level), compared

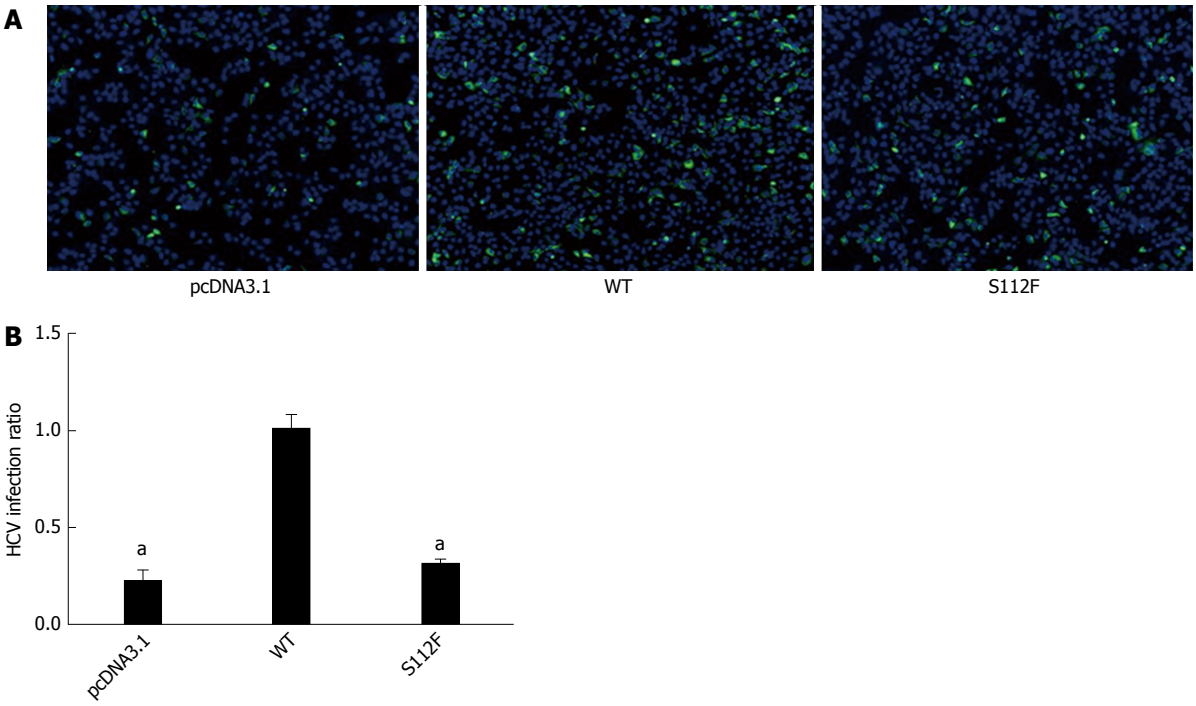

Figure 2 Effect of the class B scavenger receptor I single amino acid mutant on hepatitis C virus infectivity. A: Immunofluorescence assay (IFA) of the effects of the SR-BI single amino acid mutant on hepatitis C virus (HCV) infectivity. Huh7-siSR-BI cells were seeded in 96-well plates, cultured overnight, and then transfected with pcDNA3.1 (NC), pcDNA-SR-BI (WT), or pcDNA- SR-BI/S112F. HCVcc (10^3 FFU/mL) was added 24 h after transfection, and the IFA was performed 48 h later. B: Effect of the SR-BI single amino acid mutant on the HCV viral RNA. Huh7-siSR-BI cells were seeded in 24-well plates, cultured overnight, and then transfected with pcDNA3.1 (NC), pcDNA-SR-BI, or pcDNA-SR-BI/S112F. HCVcc (10^4 FFU/mL) was added 24 h after transfection, and cells were harvested 72 h later to prepare RNA for the qRT-PCR analysis ($^aP < 0.05$). SR-BI: Class B scavenger receptor I; WT: Wild type.

with pcDNA3.1 vector control and pcDNA-SR-BI WT control (Figure 2B).

S112F mutation decreases the expression of the SR-BI mRNA and protein

Huh7-siSR-BI cells were transfected with pcDNA3.1, pcDNA-SR-BI and pcDNA-SR-BI/S112F. Forty-eight hours after transfection, cells were harvested and analyzed by Western blot and qRT-PCR, and the results showed that the levels of the SR-BI S112F mRNA and protein were decreased compared with SR-BI WT cells (Figure 3).

SR-BI S112F mutation impairs the binding of SR-BI to the HCV E2 protein

SR-BI has been reported to bind to HCV E2 protein. The ability of CHO cells expressing WT and mutant SR-BI to bind to the HCV E2 protein that had been expressed in HEK 293T cells was analyzed, and the results showed that the S112F mutation impaired the binding of SR-BI to the HCV E2 protein (Figure 4). We also performed the binding assay using HCVcc instead of the expressed HCV E2 protein and obtained similar results.

SR-BI S112F mutation decreases HDL uptake by SR-BI

We performed the HDL uptake assay with DiI-HDL to determine whether the single amino acid mutation affects the HDL uptake ability of SR-BI. Huh7-siSR-BI cells were first transfected with SR-BI WT and mutant S112F plasmids, and then DiI-HDL was added. Based on the microscopic images, the S112F mutation decreased HDL uptake compared with SR-BI WT (Figure 5).

DISCUSSION

The SR-BI receptor is associated with lipid metabolism and participates in the bidirectional transport of cholesterol between cells and HDL. Increased clearance of HDL-CE from plasma and enhanced reverse cholesterol transport have been shown to significantly reduce atherosclerosis in animal models[23]. Previous studies have confirmed that the extracellular domain of SR-BI is critical for its receptor function[19].

Recently, the functions of SNPs in the SR-BI gene have been studied both in human and animal models. Acton and colleagues were the first to identify the associations between SNPs in the SR-BI gene and plasma lipid levels and body mass index in a Caucasian European population[33]. The nonsynonymous exon 1 SNP (rs4238001 [G2S]), which encodes a different amino acid, was significantly associated with higher HDL cholesterol (HDL-C) and lower LDL cholesterol (LDL-C) levels[33]. In 2011, a P297S missense mutation in SR-BI was reported, and people who carry the P297S mutation have increased HDL cholesterol levels[26]. Then, two novel missense mutations, S112F and

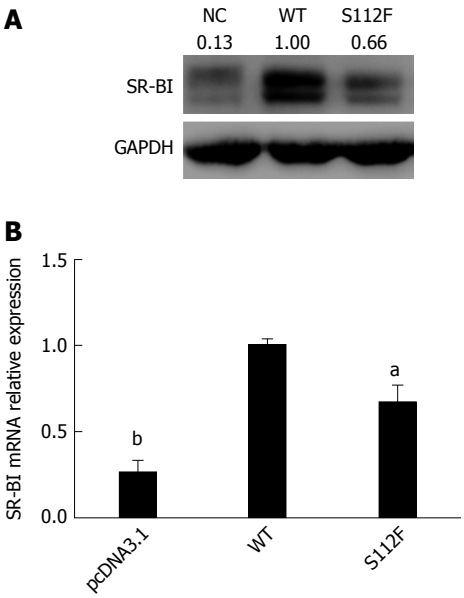

Figure 3 Effects of the single amino acid mutant on class B scavenger receptor I mRNA and protein expression. A: Effect of the single amino acid mutant on SR-BI protein expression. Huh7-siSR-BI cells were seeded in 24-well plates, cultured overnight, and transfected with pcDNA3.1 (NC), pcDNA-SR-BI, or pcDNA-SR-BI/S112F. Cells were harvested 72 h after transfection and lysed with RIPA cell lysis buffer; Western blot was performed to analyze the expression of the SR-BI protein. B: Effect of the single amino acid mutant on the SR-BI mRNA level. Huh7-siSR-BI cells were seeded in 24-well plates, cultured overnight, and then transfected with pcDNA3.1 (NC), pcDNA-SR-BI (WT), or pcDNA-SR-BI/S112F (S112F). Cells were harvested 72 h after transfection and RNA was isolated and the level of the SR-BI mRNA was analyzed using qRT-PCR ($^{a}P < 0.05$; $^{b}P < 0.001$). SR-BI: Class B scavenger receptor I; WT: Wild type.

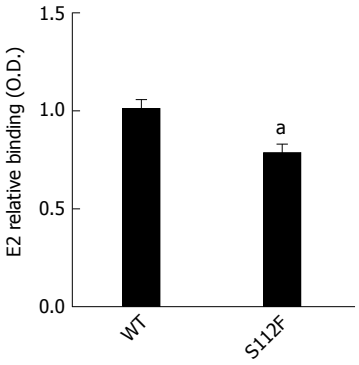

Figure 4 Ability of the class B scavenger receptor I single amino acid mutant to bind to the hepatitis C virus E2 protein. CHO cells expressing the wild type (WT) and mutant SR-BI protein were incubated with equivalent amounts of hepatitis C virus E2 protein that had been expressed in HEK 293T cells for 1 h at RT, washed twice, and incubated with an anti-E2 mAb for 1 h at RT. After labeling with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen), the mean fluorescence intensity was quantified by flow cytometry ($^{a}P < 0.05$). SR-BI: Class B scavenger receptor I.

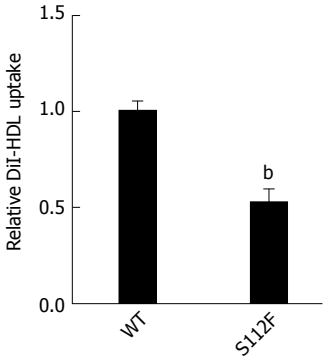

Figure 5 Effect of the single amino acid mutant on high-density lipoprotein absorption by class B scavenger receptor I. Huh7-siSR-BI cells were seeded in a 96-well plate and cultured overnight prior to transfection. Cells were transfected with the SR-BI wild type (WT) and mutant S112F plasmids using LipofectamineTM. Complete culture medium was replaced 6 h after transfection and cells were cultured for 48 h before DiI-HDL (200 μg/mL) was added, and the cells were cultured for an additional 4 h. Nuclei were stained with Hoechst for 10 min, and cells were observed and images captured using a microscope ($^{b}P < 0.01$). SR-BI: Class B scavenger receptor I.

T175A, in human SR-BI were identified in patients with atherosclerosis, which led to elevated HDL cholesterol levels[27]. A coding variant in SR-BI (I179N) significantly increased atherosclerosis, although the mutation did not dramatically affect the plasma lipid levels[34]. The hydrophobicity of N-terminal half of the extracellular domain of SR-BI was proven to be critical for the SR-BI-mediated cholesterol transport function, and this domain might function by interacting with other integral membrane proteins[35-37]. S112F, T175A and I179N point mutations occurred in the same region, but had different effects on the function of SR-BI and require further study.

HCV infection is a complicated process and is closely correlated with host lipid metabolism. In the attachment step, the HCV lipoviral particle (LVP) is recruited and binds to glycosaminoglycans on heparan sulfate and low-density lipoprotein receptor on host cells; then, HCV enters hepatocytes by interacting with several host entry factors, including CD81, SR-BI, the tight junction proteins CLDN1 and OCLN, and the cholesterol absorption receptor Niemann-Pick C1-like-1[38]. Lipids and lipid receptors play key roles in the early stage of HCV infection, and researchers have postulated that LVP was actually endocytosed into the hepatocytes as a regular lipoprotein[39].

During the entry step, the HCV E2 HVR1 region interacts with the extracellular loop of SR-BI in both the binding and post-binding steps[40,41]. Therefore, strategies targeting SR-BI have been reported to inhibit HCV infection. SR-BI binds to serum amyloid A (SAA), an acute-phase protein produced by the liver, promoting SAA internalization and inhibiting HCV entry[42]. A small-molecule antiviral compound, ITX5061, has been reported to impede the uptake of HDL by SR-BI and blocks the uptake of HCV viral particles by hepatocytes[43,44].

In addition to the entry step, the replication of HCV in the membrane web and release from hepatocytes are associated with the host lipid metabolism. Since SR-BI has a key role in the host lipid metabolism and SNPs in the SR-BI gene have been reported to modulate the function of SR-BI, we studied the effect of the S112F missense mutation in SR-BI on

the infectivity of HCVcc in SR-BI knock-down Huh7 cells. The Huh7-siSR-BI cell line was established by screening Huh7 cells with puromycin after infection with Lv-SR-BI-shRNA[29]. We first constructed SR-BI S112F using site-directed mutagenesis, and then Huh7-siSR-BI cells were used to detect the effects of the single amino acid mutant in SR-BI on the entry and infectivity of HCV. The S112F single amino acid mutation decreased HCV entry and the infection of HCVcc compared with SR-BI WT. We further assessed the effects of S112F on the SR-BI mRNA and protein levels to determine how the S112F mutation affected SR-BI, and the results showed that the levels of both the SR-BI mRNA and protein decreased when Ser112 was replaced with Phe. Then, we detected the ability of SR-BI S112F to bind to HCV E2 and showed that the binding ability decreased, potentially due to the decreased level of the SR-BI protein. Finally, since the S112F mutation is associated with an abnormal HDL level and HCV replication is closely correlated with lipid metabolism, we measured the HDL uptake ability of SR-BI S112F and observed a decrease compared with SR-BI WT.

We performed a literature search to determine why the S112F mutant significantly decreased the expression of SR-BI and found that Ser112 is located in the extracellular domain of SR-BI. SR-BI and LIMP II belong to the same family and share 34% sequence identity and 56% sequence homology. The X-ray crystal structure of the extracellular domain of human LIMP II has been solved. Therefore, we used the LIMP II structure as a guide to generate a homology model of human SR-BI. Ser112 in SR-BI is located in a hydrophilic pocket, which is conserved in SR-BI. If the serine (hydrophilic amino acid) is mutated to phenylalanine (hydrophobic amino acid), this hydrophilic pocket will be destroyed. Thus, the protein will not fold correctly, which might be responsible for the downregulation of SR-BI expression in cells expressing the SR-BI S112F mutant.

In summary, we constructed the S112F single amino acid SR-BI mutant and analyzed the effects of this mutant on HCV entry and infectivity. The S112F single amino acid mutant decreased the levels of the SR-BI mRNA and protein and subsequently reduced the binding of SR-BI to HCV E2 protein, as well as the SR-BI-dependent HCV entry and infectivity of HCVcc. In this study, Huh-7-siSR-BI cells expressing SR-BI S112F also showed decreased HDL uptake, but the effects of this mutation on the release of progeny viruses require further investigation.

COMMENTS

Background

Human class B scavenger receptor I (SR-BI) is an important receptor associated with host lipid metabolism, and both SR-BI itself and its ligands also play crucial roles in the life cycle of hepatitis C virus (HCV). Single nucleotide polymorphisms (SNPs) in the host genome that affect the virus-host interaction have received increasing attention in recent years. SNPs in the *IL28B* gene are associated with HCV prognosis.

Research frontiers

SNPs in the SR-BI gene are reported to be associated with the elevated plasma HDL-cholesterol levels and virological response of HCV-infected patients. However, the relation between SR-BI SNPs and HCV infectivity has not yet been clearly identified.

Innovations and breakthroughs

This study is the first to evaluate the effect of the single amino acid polymorphism SR-BI S112F on the infectivity of hepatitis C virus derived from cell culture (HCVcc) using a cell culture model. SR-BI S112F inhibited HCVcc infectivity by downregulating the expression of the SR-BI protein.

Applications

Based on the results from the present study, the SR-BI S112F single amino acid mutant affected both HCV entry and infectivity. Future studies of SR-BI SNPs will provide insights into the host-virus interaction and will help identify new treatments for HCV infection.

Terminology

HCVpp, referred as HCV pseudoparticles, were prepared by inserting HCV envelope proteins into pLenti6 transfer vector, and transfected into HEK 293T cells together with helper plasmids Gag/Pol and Rev. HCVcc, referred as real HCV virus particles in cell culture, is prepared by transfection of whole genomic HCV RNA transcripts into Huh 7 or its derived cell lines.

Peer-review

The manuscript is well written. Authors demonstrated that single amino acid polymorphism of SR-BI S112F decreased the infectivity of HCVcc in SR-BI silenced Huh 7 cells. This is the first study evaluating the effect of SR-BI S112F on the infectivity of HCV even though in the *in vitro* cell culture system. The study showed that SNPs of SR-BI were critical for HCV infectivity.

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ORIGINAL ARTICLE

Basic Study

Fibroblast-derived CXCL12/SDF-1α promotes CXCL6 secretion and co-operatively enhances metastatic potential through the PI3K/Akt/mTOR pathway in colon cancer

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Abstract

AIM

To investigate the underlying mechanism by which CXCL12 and CXCL6 influences the metastatic potential of colon cancer and internal relation of colon cancer and stromal cells.

METHODS

Western blotting was used to detect the expression of CXCL12 and CXCL6 in colon cancer cells and stromal cells. The co-operative effects of CXCL12 and CXCL6 on proliferation and invasion of colon cancer cells and human umbilical vein endothelial cells (HUVECs) were determined by enzyme-linked immunosorbent assay, and proliferation and invasion assays. The angiogenesis of HUVECs through interaction with cancer cells and stromal cells was examined by angiogenesis assay. We eventually investigated activation of PI3K/Akt/mTOR signaling by CXCL12 involved in the metastatic process of colon cancer.

RESULTS

CXCL12 was expressed in DLD-1 cancer cells and fibroblasts. The secretion level of CXCL6 by colon cancer cells and HUVECs were significantly promoted by fibroblasts derived from CXCL12. CXCL6 and CXCL2 could significantly enhance HUVEC proliferation and migration ($P < 0.01$). CXCL6 and CXCL2 enhanced angiogenesis by HUVECs when cultured with fibroblast cells and colon cancer cells ($P < 0.01$). CXCL12 also enhanced the invasion of colon cancer cells. Stromal cell-derived CXCL12 promoted the secretion level of CXCL6 and co-operatively promoted metastasis of colon carcinoma through activation of the PI3K/Akt/mTOR pathway.

CONCLUSION

Fibroblast-derived CXCL12 enhanced the CXCL6 secretion of colon cancer cells, and both CXCL12 and CXCL6 co-operatively regulated the metastasis *via* the PI3K/Akt/mTOR signaling pathway. Blocking this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer.

Key words: CXCL12/SDF-1α; CXCL6; Metastasis; PI3K/Akt/mTOR pathway; Colon cancer

Core tip: This study has provided the first report of fibroblast-derived CXCL12 enhancement of CXCL6 secretion in colon cancer cells, and of both CXCL12 and CXCL6 co-operatively regulating metastasis through the PI3K/Akt/mTOR signaling pathway. Blockage of this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer. Our work might encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

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INTRODUCTION

Colon cancer is the fourth most frequently diagnosed cancer in the United States. In 2015, an estimated 93090 new cases of colon cancer occurred in the United States. During that same year, it was estimated that 49700 patients died from colon and rectal cancers[1]. The poor prognosis of colon cancer is attributable to its tendency of metastases. However, the precise mechanisms that determine the directional proliferation and invasion of cancer cells into specific organs remain to be established[2,3]. Therefore, exploring the fundamental mechanism of invasion, proliferation, metastasis and tumor biological behaviors at the level of cellular or molecular microenvironments is needed in clinical diagnosis and therapy.

Chemokines (chemotactic cytokines) form a complex family of small, secreted proteins that play an important role in innate and adaptive immunity, homeostatic processes, angiogenesis and tumorigenesis[4,5]. Based upon the position of conserved cysteine residues, chemokines are classified into four subfamilies (C, CC, CXC, CX3C)[6]. CXC chemokines have been proven to modulate tumor behaviors, especially in regulation of angiogenesis, activation of a tumor-specific immune response and stimulation of tumor cell proliferation in an autocrine or paracrine fashion[7]. However, updated research has shed new light on this subfamily of cytokines, indicating that its members have multifaceted roles in the microenvironment that consists of the tumor cells themselves and/or stromal cells, including infiltrating leukocytes, endothelial cells (ECs) and fibroblasts.

The functions of CXC chemokines in the tumor microenvironment depend considerably on the chemokine type and tumor and stromal cells' characteristics. In addition, there are cases in which chemokines have been implicated as having tumor-inhibiting gene activities, and there are many more examples of CXC chemokines with tumor-promoting roles[8-11]. Two of the most famous members are the stromal cell-derived factor-1 (SDF-1/CXCL12/IL12) and chemokine ligand 6 (CXCL6). Numerous studies have shown that their activities would increase the establishment of tumorigenesis, invasion, proliferation and metastases.

Recent analysis has shown that CXCL12 supports the survival or growth of a variety of normal or malignant cell types, including hematopoietic progenitors, germ cells, leukemia B cells and breast carcinoma cells[12-15]. Other studies have shown that the CXCL12/CXCR4 and related axis are involved in tumor metastasis to sites which are characterized by high production of CXCL12, such as liver, lung and bone marrow[16,17]. Activation of the CXCL12/CXCR4 signaling axis leads to chemotaxis, cell survival, and/or proliferation; however, the downstream signaling cascades are tissue-specific and not well characterized in EC[18].

CXCL6, a small cytokine belonging to the CXC chemokine family, is also known as granulocyte chemotactic protein 2. As its former name suggests, CXCL6 is a chemoattractant for neutrophilic granulocytes[13-14]. It elicits its chemotactic effects by interacting with the chemokine receptors CXCR1 and CXCR2. This tumor progression may occur as a function of the regulation of angiogenesis, cell motility, immune cell infiltration, cell growth and survival in the microenvironment, and modulation of local anti-

tumor immune responses[19]. As evidenced by various experiments, CXCL6 is over expressed in colorectal, breast, lung and thyroid cancers. Actions of tumor cells in the microenvironment were also regulated by complicated molecular mechanisms[20-23].

Different chemokines played their specific roles. Both the angiogenesis-promoting effect of CXCL6 and chemotactic effect of CXCL12 play important roles in tumorigenesis and metastasis[24,25]. However, the molecular mechanisms of the active signaling pathway by which CXCL12 and CXCL6 co-operatively regulate metastasis of colon cancer remain to be clarified.

The purpose of this study was to investigate the co-operative promotion of metastatic potential and the underlying mechanism of CXCL12 and CXCL6 in order to better understand the interaction between colon cancer cells and stromal cells. Furthermore, our study provided data to demonstrate that phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway plays an important role in CXCL12 simulation and that this process is involved in the development and metastasis of colon cancer. Understanding the biologic mechanisms responsible for regulation of chemokines may enable better molecular targeted therapies to treat patients with metastatic colon cancer.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human CXCL6 and CXCL12 were purchased from R&D Systems (Minneapolis, MN, United States). Neutralizing monoclonal anti-human CXCL12 (anti-CXCL12 Ab), anti-human CXCL6 (anti-CXCL6 Ab), anti-human CXCR4 (anti-CXCR4 Ab) were obtained from Carbiochem (San Diego, CA, United States).

Cell lines and culture condition

The human colon cancer cell lines HT29, WiDr, CaCo-2, DLD-1 and Colo320 were obtained from the American Type Culture Collection (Rockville, MD, United States). DLD-1, WiDr and CaCo-2 were maintained in minimum essential medium (Eagle's; (Sigma Chemical Co., St. Louis, MO, United States) with high glucose and 10% fetal bovine serum (FBS). HT-29 and Colo320 were maintained in RPMI-1640 medium (Sigma Chemical Co.) supplemented with 10% FBS. HUVECs were obtained from Kurubo Co. (Osaka, Japan) and maintained in HuMedia-EG2 medium supplemented with 2% FBS, 5 ng/mL basic fibroblast growth factor, 10 μg/mL heparin, 10 ng/mL epidermal growth factor, and 1 μg/mL of hydrocortisone, according to the supplier's instruction (Kurubo Co.). All cells were incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 in air.

Western blot analysis

Cells were cultured in the media supplemented with 1% FBS for 1 d. After the indicated treatments, the cells were lysed in lysis buffer [25 mmol/L Tris (pH 7.8) with H_3PO, 2 mmol/L CDTA, 10 mmol/L DTT, 10% glycerol, 1% Triton® X-100, 2 mmol/L PMSF, 1 mmol/L sodium orthovanadate, and 10 μmol/L leupeptin]. The protein concentrations were measured with a BCA protein assay kit (Pierce, Rockford, IL, United States). The amounts of samples were 30 μg per lane. The lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membrane (Immobilo PVDF; Nihon Millipore Ltd, Tokyo, Japan). The membrane was incubated in the blocking buffer for 60 min at room temperature. The blocking buffer consisted of 5% nonfat dry milk dissolved into Tris-buffered saline containing 0.1% Tween 20 (TBS-T). After washing the membrane with TBS-T, the membrane was immunoblotted with each primary antibody (Ab) diluted into 1:1000-2000 overnight at 4 ℃. Afterward, membranes were washed with TBS-T three times, and subjected to HRP-conjugated secondary Ab for 60 min at room temperature. Protein Ab complexes were visualized with an ECL western blotting detection and analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom). β-actin western blots served as controls.

Enzyme-linked immunosorbent assay

All cancer cell lines and fibroblasts were separately seeded at a density of 3×10^5 cells/mL into 12-well plates containing medium with 10% FBS and allowed to adhere overnight. The medium was exchanged, and cells were cultured for an additional 48 h. The medium was collected and microcentrifuged at 1500 rpm for 5 min to remove particles, and the supernatants were frozen at -80 ℃ until performance of enzyme-linked immunosorbent assay (ELISA). Concentration of CXCL6 was measured by ELISA kit (R&D Systems) according to the manufacturer's instructions.

In order to further investigate the synergistic effect of the tumor-stromal interaction, we tested the effect of fibroblast-derived CXCL12 on cancer cell CXCL6 production using a double-chamber method in 24-well plates. Fibroblasts were seeded at a density of 1×10^5 cells/well into 24-well plates, and allowed to adhere overnight. The medium was exchanged with or without CXCL12 Ab, and then co-cultured with 5×10^4 HT-29, WiDr, CaCo-2, DLD-1 and fibroblasts cells that had been placed into inserts with 0.45-mm^2 pores (Kurabo Co.). The co-culture systems were incubated for an additional 48 h, and CXCL6 concentration was subsequently measured as described above. Each condition was assessed using 5 independent samples.

Proliferation assay

To confirm the effect of chemokines on HUVEC proliferation, we performed the proliferation assay according to the manufacturer's instructions. HUVECs were seeded at a density of 5×10^3 cells/100 μL in

96-well plates and allowed to adhere overnight. Then, cultures were re-fed with fresh media containing various concentrations of CXCL6 or CXCL12. After 72 h incubation, 10 μL WST-1 reagent was added to each well and cells were incubated for another 4 h at 37 ℃, then the cell proliferation was measured by the WST-1 Cell Proliferation Assay System (Takara Bio Inc., Shiga, Japan). The absorbance was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, United States) at a test wavelength of 450 nm and reference wavelength of 690 nm.

Invasion assay

The effects of CXCL12, CXCL6 and co-cultures with fibroblasts or colon cancer cells (DLD-1) on invasive capability of HUVECs were determined by Matrigel-coated invasion chambers (Becton Dickinson, Bedford, MA, United States) according to the manufacturer's instructions. This system is separated by a PET membrane coated with Matrigel Matrix such that only invasive cells can migrate through the membrane to the reverse side. HUVECs (5×10^4 cells/mL) were suspended in medium containing 2% FBS and seeded into the Matrigel pre-coated transwell chambers consisting of polycarbonate membranes with 8-μm pores, and fibroblasts or DLD-1 cells were seeded at a density of 2×10^5 cells/well into the inner chambers in 24-well plates, then the transwell chambers were then placed into 24-well plates, into which we added basal medium only or basal medium containing gradient concentrations of CXCL6 (0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 10 ng/mL + 10 μg/mL CXCL6 Ab) or CXCL12 (0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 10 ng/mL + 10 μg/mL CXCL12 Ab). After incubating for 24 h and HUVECs for 16 h, the upper surface of the transwell chambers was wiped with a cotton swab and the invading cells were fixed and stained with Diff-Quick stain. The number of invading cells was counted in five random microscopic fields of the low filter surface under a microscope at 200 × magnification. Each condition was assessed in triplicate.

Angiogenesis assay

To investigate the influence of CXCL6 on tubule formation by HUVECs, HUVECs and fibroblasts were co-culture in the basal medium using an angiogenesis kit (Kurabo Co.) according to the manufacturer's protocol. First, HUVECs and fibroblasts were co-cultured in 24-well plates with basal medium. The media were exchanged every 2 d, with co-incubation continuing for a total of 11 d. The co-culturing system was stained with anti-CD31 Ab. The areas of angiogenesis were measured quantitatively over ten different microscopic fields for each well using an image analyzer (Kurabo Co.).

Angiogenic activity during co-culture with colon cancer cells in vitro

To further investigate the influence of colon cancer cell-derived CXCL12 on tubular formation by HUVECs, the colon cancer cells (DLD-1 secreting CXCL12 or CaCo-2 and HT-29 not secreting CXCL12), HUVECs, and fibroblasts were co-cultured using a double-chamber method in 24-well plates. DLD-1, CaCo-2 or HT-29 cells (5×10^4 cells) were seeded into transwell chambers, consisting of polycarbonate membrane with 0.45-m pores and allowed to adhere overnight. Transwell chambers were then placed in the HUVECs/fibroblasts co-culture system with or without 10 ng/mL of CXCL12 or CXCL12 Ab and exchanged on the sixth day. All cells were cultured for a total of 11 d. HUVEC tubular formation was described as above. This assay allowed us to evaluate angiogenesis quantitatively and examine tumor-stromal interactions through soluble cytokines.

Statistical analysis

Data are presented as mean ± SD. Differences in the mean of two groups were analyzed by an unpaired *t*-test. Multiple group comparisons were performed by one-way ANOVA with a post hoc test for subsequent individual group comparisons. $P < 0.05$ was considered statistically significant. Mean values and SD were calculated for experiments performed in triplicate (or more).

RESULTS

Expression of CXCL12, CXCL6 and CXCR4 proteins in colon cancer cell lines and stromal cells

Western blotting results revealed that CXCL12 protein was only expressed in fibroblasts and DLD-1, but not in HT29, WiDr, CaCo-2, Colo320 and HUVECs. CXCR4 and CXCL6 were expressed in all colon cancer cell lines, fibroblasts and HUVECs (Figure 1).

Effect of CXCL12 on secreted level of CXCL6 from colon cancer cell lines and HUCVECs

The secreted CXCL6 level was measured by ELISA assay in colon cancer cell lines and stromal cells. On the basis of this assay, secretion of CXCL6 was higher in DLD-1 and HT-29 cell supernatants than in supernatants from CaCo-2, WiDr and HUVECs. The addition of recombinant CXCL12 significantly enhanced CXCL6 production in CaCo-2 (2.54-fold *vs* control, $P < 0.01$; Figure 2A), WiDr (2.07-fold *vs* control, $P < 0.01$; Figure 2B), HT-29 (1.87-fold *vs* control, $P < 0.01$; Figure 2C) and HUVEC (2.79-fold *vs* control, $P < 0.01$; Figure 2E). Likewise, co-culture with fibroblast cells also significantly enhanced CaCo-2 (1.89-fold *vs* control, $P < 0.01$), WiDr (1.67-fold *vs* control, $P < 0.01$), HT-29 (1.62-fold *vs* control, $P < 0.01$) and HUVEC (2.15-fold, *vs* control, $P < 0.01$) cells' secretion of CXCL6. On the other hand, recombinant CXCL12 and co-culture with fibroblasts did not promote the CXCL6 secretion in DLD-1 culture supernatants (Figure 2D). Co-culture with DLD-1 cells significant

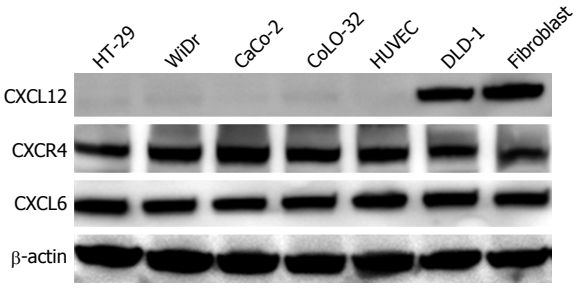


Figure 1 Expression levels of stromal cell-derived factor-1, CXC chemokine receptor 4 and granulocyte chemotactic protein-2 in colon cancer cell lines and stromal cells. The protein expression levels of CXCL2, CXCR4 and CXCL6 in colon cancer cell lines and stromal cells were determined in whole-cell lysates by western blotting analysis. Thirty micrograms of total cell lysate were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was probed with antibodies to CXCL12, CXCR4 and CXCL6. β-actin was used as a loading control. CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; CXCR4: CXC chemokine receptor 4.

enhanced CXCL6 secretion level in the HUVEC culture supernatants as well ($P < 0.01$), because fibroblasts could secrete CXCL12 protein. Furthermore, the enhanced CXCL6 production elicited by co-culturing with fibroblast cells and recombinant CXCL12 were significantly inhibited in the presence of CXCL12 Ab ($P < 0.01$).

HUVEC proliferation following treatment with CXCL6, CXCL12 and fibroblast cell-cultured supernatants

To create stromal cell supernatants, fibroblast cells were seeded to a final number of 5×10^6 cells/5 mL into 100-mm dishes containing medium with 10% FBS, and were cultured overnight. Cells were then cultured in medium containing 2% FBS for 48 h. The culture media were collected and microfuged at 1500 rpm for 5 min to remove any particles, and the supernatants were used in proliferation assays. Recombinant CXCL6 elicited enhanced proliferation of HUVECs in a dose-dependent manner, and co-culture with fibroblasts caused significantly enhanced HUVEC proliferation ($P < 0.05$, $P < 0.01$; Figure 3A). Recombinant CXCL6 also promoted the proliferation of HUVECs in a concentration-dependent manner ($P < 0.05$, $P < 0.01$; Figure 3B).

CXCL6 and CXCL6 promotion of colon cancer cell and HUVEC invasiveness

The invasion assay was used to investigate whether CXCL12 and CXCL6 influence invasiveness of colon cancer cell lines. The invasive capacity of HT-29 cells was promoted by stimulation using recombinant CXCL6 (Figure 4A) and CXCL12 (Figure 4B) in a concentration-dependent manner ($P < 0.05$, $P < 0.01$), and 10 ng/mL of CXCL6 and CXCL12 significantly promoted cancer cell invasion ($P < 0.01$). Interestingly, CXCL6 (Figure 4C) and CXCL12 (Figure 4D) also significantly enhanced the invasion of HUVECS in a dose-dependent manner ($P < 0.05$, $P < 0.01$). However, the invasive behavior of HUVECs upon CXCL6 stimulation was more significant than upon CXCL12 stimulation. Enhancement of invasive ability of HUVECs by CXCL6 and CXCL12 stimulation were blocked by pre-incubating HUVECs with neutralizing anti-CXCL6 and anti-CXCL12 Ab, respectively ($P < 0.05$, $P < 0.01$; Figure 4C).

Effect of co-culturing with fibroblasts and DLD-1 cells on HUVEC invasiveness

To investigate the interaction between colon cancer and stromal cell-derived CXCL12 in the tumor microenvironment, we next examined the role of cell-derived CXCL12 on HUVECs' invasiveness using the Matrigel double culturing chamber invasion assay. The invasive capability of HUVECs were enhanced by co-cultivation with fibroblasts ($P < 0.01$; Figure 4E) and DLD-1 ($P < 0.01$; Figure 4F); meanwhile, the enhancement of HUVEC invasive behavior was inhibited by neutralizing anti-CXCL12 Ab ($P < 0.01$), and the addition of recombinant CXCL6 significantly enhanced HUVECs invasiveness in co-cultivation with fibroblasts system as well ($P < 0.01$; Figure 4E). At the same time, co-cultivation with CaCo-2 cells did not significantly increase the invasion of HUVECs.

CXCL6 and CXCL12 enhancement of tube formation

To further determine the role of CXCL12 and CXCL6 in the living cell microenvironment, we focused on the interaction between tumor cells and stromal cells by characterizing angiogenic activity in co-cultured fibroblasts and vascular ECs, and the effect of CXCL6 and CXC12 in this system. Initially, we measured the influence of CXCL6 and CXCL12 on tube formation by HUVECs. HUVEC tube formation was significantly enhanced in a dose-dependent manner following treatment CXCL6 ($P < 0.01$; Figure 5A) and CXCl12 ($P < 0.01$; Figure 5B). The enhanced angiogenesis of HUVECs was inhibited by the addition of neutralizing anti-CXCL6 and anti-CXCL12 Ab ($P < 0.01$).

Effect of colon cancer cells with or without CXCL12 on tube formation by HUVECs

In order to explore the different secreted CXCL2 of colon cancer cells influence on tube formation by HUVECs, we cultured three cell lines using double chamber methods to determine the interaction among them. The tubular formation was significantly enhanced by co-culture with DLD-1 cells compared with control (HUVECs and fibroblasts only) or co-culture with HT-29 and CaCo-2 cells, respectively ($P < 0.01$; Figure 5C). Moreover, the CXCL12 and CXCL6 could significantly promote the tubular formation in co-culture with HT-29 and CaCo-2 cells system ($P < 0.01$). In contrast, the enhanced tubular formation by HUVECs was significantly inhibited by addition of anti

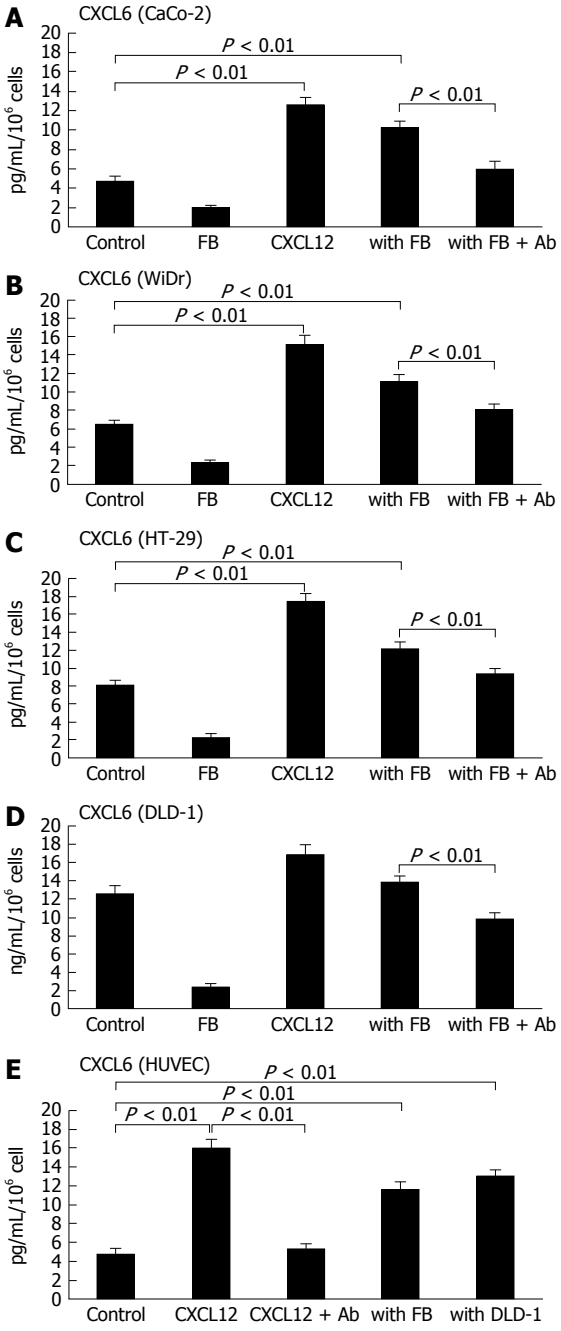


Figure 2 Enhancement of secreted granulocyte chemotactic protein-2 levels in colon cancer cell lines and stromal cells by recombinant stromal cell-derived factor-1 and co-culture with fibroblasts. The alteration of CXCL6 secretion from colon cancer cell lines [CaCo-2 (A), WiDr (B), HT-29 (C) and DLD-1 (D)] by recombinant CXCL12 stimulation or co-culture with fibroblasts (FB) were determined by enzyme-linked immunosorbent assay in cell culture medium. Meanwhile, colon cancer cells were treated with anti-CXCL12 antibody (Ab) for 2 h, and the concentration of CXCL6 was measured by ELISA in supernatants from colon cancer cells. Effect on secretion of CXCL6 from HUVECs stimulated by recombinant CXCL12 in co-culture system with fibroblasts and the colon cancer cells DLD-1 are shown (E). The experimental detail is described in the "Materials and Methods" section. Control: colon cancer cells only; FB: fibroblasts only; CXCL12: treated with recombinant CXCL12; with FB: colon cancer cells co-cultured with fibroblasts; with FB + Ab: colon cancer cells co-cultured with fibroblasts and pre-treated with anti-CXCL12 Ab. The values are expressed as mean ± SD. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

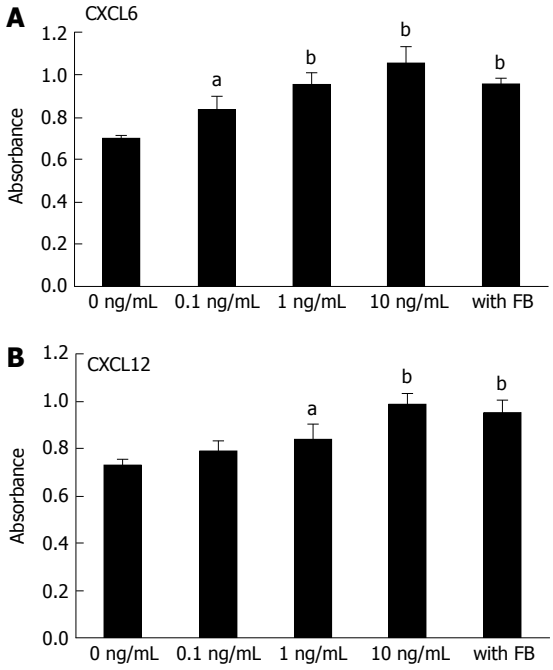
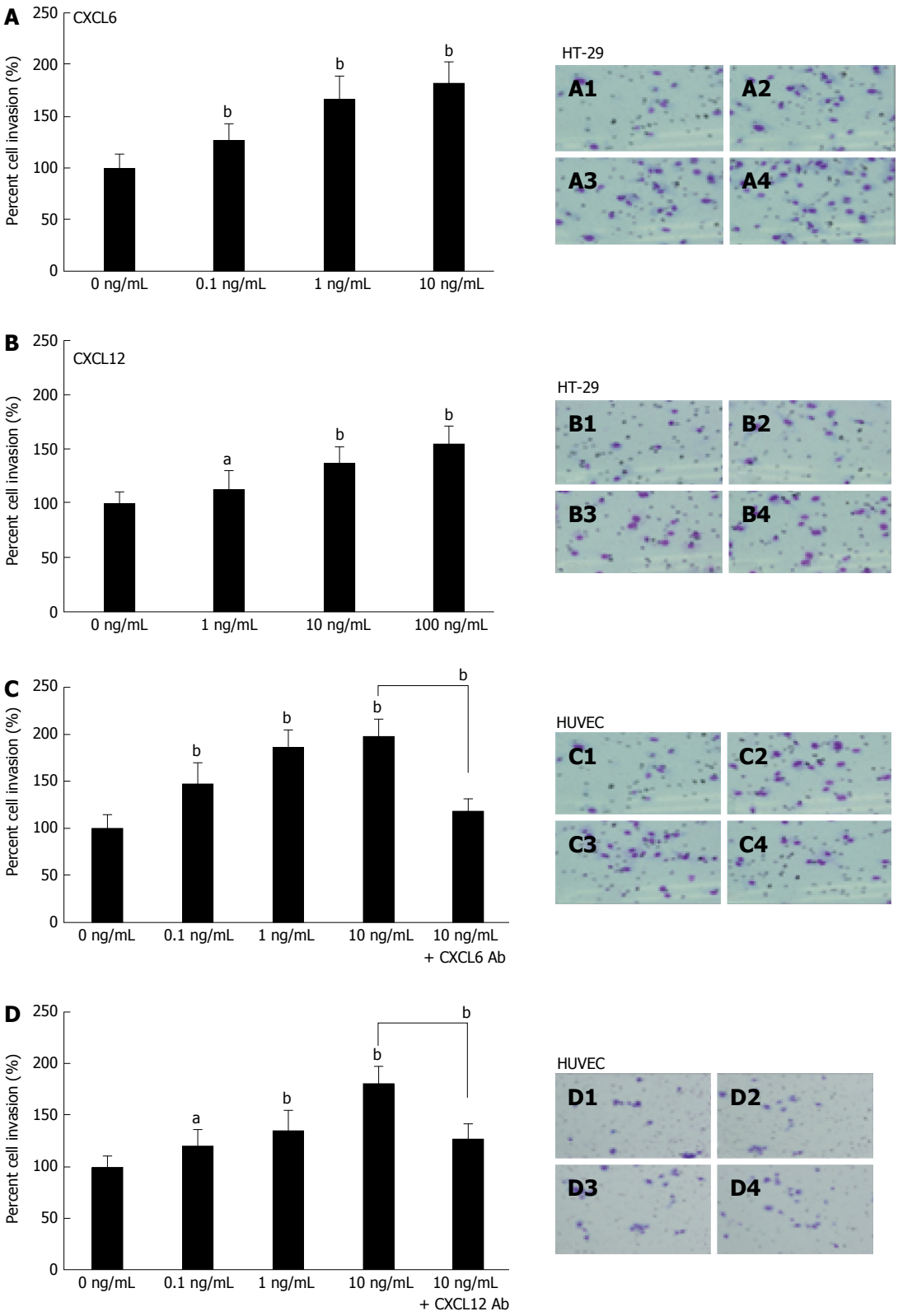


Figure 3 Effect of stromal cell-derived factor-1, granulocyte chemotactic protein-2 and conditioned medium from fibroblasts on human umbilical vein endothelial cell proliferation. HUVECs were cultured in medium containing different concentrations of CXCL6 (A), CXCL12 (B) and conditioned medium from fibroblasts. After 72 h of incubation, HUVEC proliferation was assessed using premixed WST-1 cell proliferation assay (column mean absorbance reading; Bars = SD). Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{a}P < 0.05$, $^{b}P < 0.01$. CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

CXCL12 Ab in co-culture with DLD-1 cells ($P < 0.01$).

Activation of the PI3K/Akt/mTOR signaling pathway after CXCL12 stimulation in colon cancer and stromal cells

We used the colon cancer cell HT-29 and stromal cell HUVECs to examine activation of the PI3K/Akt/mTOR signaling pathway, a downstream target of CXCL12. The stimulation by 10 ng/mL of CXCL12 could increase Akt (Figure 6A), PI3K (Figure 6B) and mTOR (Figure 6C) phosphorylation in a time-dependent manner in HT-29 cells and HUVECs. To determine the role of mTOR, we investigated the effect of CXL12 and PI3K/Akt inhibitor on the activation of mTOR in colon cancer cells and HUVECs; we looked at the effects of IGF-1 and/or PI3/Akt kinase inhibitors on the activation of mTOR in these cells. HT-29 and HUVECs were pre-treated for 60 min with PI3K/Akt inhibitors and then stimulated overnight with CXCL12 (100 ng/mL). The extracted proteins were separated by SDS-PAGE, transferred to membranes, and the membranes probed with Ab directed against phospho-mTOR and total mTOR. We found that by CXCL12-mediated increase phospho-mTOR was inhibited by 50 μmol/L PI3K inhibitor (LY294002) and 50 μmol/L Akt kinase inhibitor. These data indicate that CXCC12 regulates the PI3K/Akt/mTOR signaling pathway activity and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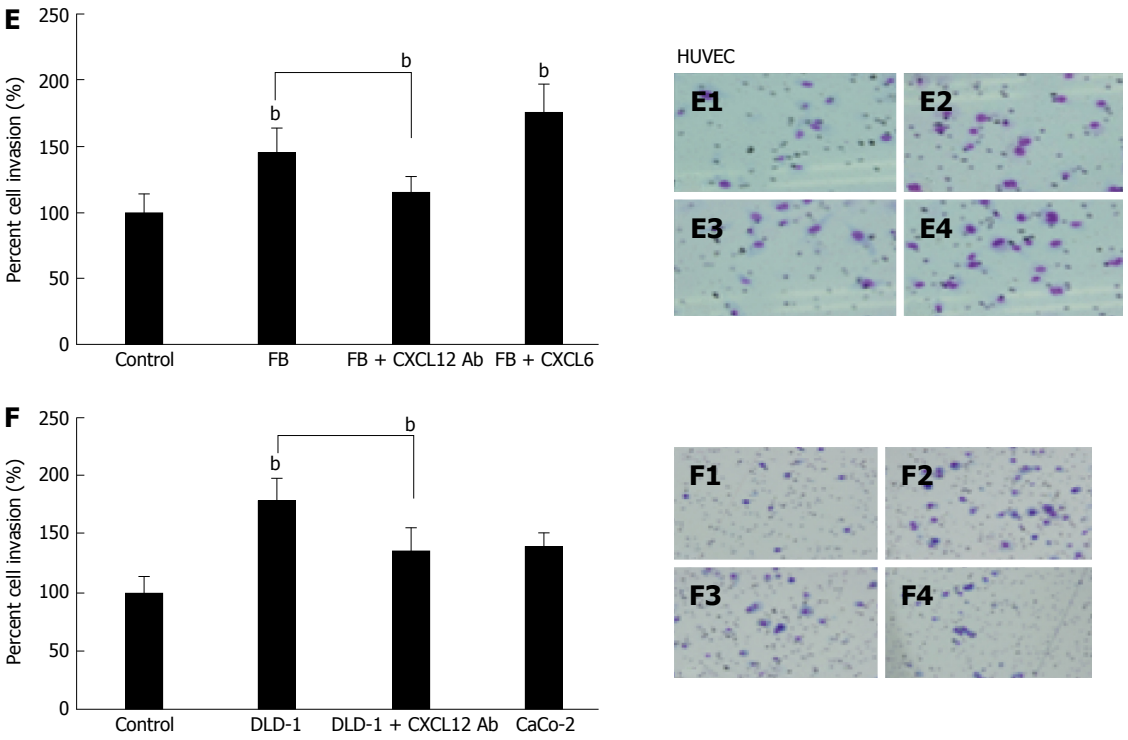

Figure 4 Effect of granulocyte chemotactic protein-2, stromal cell-derived factor-1 and co-culture with fibroblasts or DLD-1 on colon cancer cell and human umbilical vein endothelial cell invasiveness. The influence of different concentrations of CXCL6 (A), CXCL12 (B) or co-culture with fibroblasts on colon cancer cell invasiveness was measured by the BD Bio-Coat Matrigel invasion assay system (BD Biosciences). HT-29 (A and B) cells and HUVECs (C and D) were pre-treated with different concentrations of CXCL6 and CXCL12, and co-culture with fibroblasts (E) or DLD-1 (F), or pre-treated with or without anti CXCL6 or CXCL12 antibody, and following a 24-h incubation. The invading cells were fixed and stained with Diff-Quick stain. Invading cells were counted in five random microscopic fields (× 200). The experiment detail is described in the "Material and Methods" section. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{a}P < 0.05$, $^{b}P < 0.01$. A1: HT-29 cells only; A2: 0.1 ng/mL of CXCL6; A3: 1 ng/mL of CXCL6; A4: 10 ng/mL of CXCL6. B1: HT-29 cells only; B2: 0.1 ng/mL of CXCL12; B3: 1 ng/mL of CXCL12; B4: 10 ng/mL of CXCL12; C1: HUVECs only; C2: 1 ng/mL of CXCL6; C3: 10 ng/mL of CXCL6; C4: 10 ng/mL of CXCL6 treated with 10 μg/mL CXCL6 Ab. D1: HUVECs only; D2: 1 ng/mL of CXCL12; D3: 10 ng/mL of CXCL12; D4: 10 ng/mL of CXCL12 treated with 10 μg/mL CXCL12 Ab. E1: HUVECs only; E2: HUVECs co-culture with fibroblasts; E3: Co-culture with fibroblasts + 10 μg/mL CXCL12 Ab; E4: Co-culture with fibroblasts + 10 ng/mL CXCL6. F1: HUVECs only; F2: HUVECs co-culture with DLD-1 cells; F3: Co-culture with DLD-1 cells + 10 μg/mL CXCL12 Ab; F4: Co-culture with CaCo-2 cells. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

suggest that the PI3K/Akt/mTOR signaling pathway could participate in the regulation of metastatic behavior by colon cancer cells (Figure 6D).

DISCUSSION

Many tumors produce chemokines, which may explain the presence of the tumor-associated microenvironment. However, the role of these chemokines in tumor biology is still unclear. Chemokines form a complex family of small, secreted proteins that play important roles in innate and adaptive immunity, homeostatic processes, angiogenesis and tumorigenesis[4]. Recent exploration of the tumor microenvironment has become the crux of research aimed at explaining tumor behaviors, especially those involving metastasis of solid tumors as in colon, stomach, liver, lung and breast cancers.

The tumor microenvironment consists of tumor, stromal, immune and inflammatory cells, all of which produce cytokines, growth factors and adhesion molecules[26,27], and the abnormal expression of cytokines has been shown to have great effect on tumor behaviors, such as tumor progression and metastasis[28,29]. The CXC chemokine family of cytokines, which are founded in the microenvironment, represent a significant difference between tumors and normal tissues[30]. The tumor microenvironment contains secreted chemokines representing distinctive profiles, the components of each having specific target cells. The chemokine CXCL12, through its receptor CXCR4, positively regulates angiogenesis by promoting EC migration and tube formation. However, the relevant downstream signaling pathways in EC have not been defined.

Our previous studies elucidated that IL-1α is one of the most important inflammatory cytokines involved in the metastatic process of colon cancer. IL-1α contributed to the regulation of tumor growth, progression, and liver metastasis in primary gastric carcinoma and pancreatic cancer. Pancreatic cancer cell-derived IL-1α increases fibroblast-derived hepatocyte growth factor (HGF) secretion in a paracrine manner, and that enhanced HGF expression promotes invasion, proliferation and angiogenesis of cancer cells. In the living microenvironment of the tumor, the chemokines act as couriers or guides for promoting tumor development and the metastasis process[31-33].

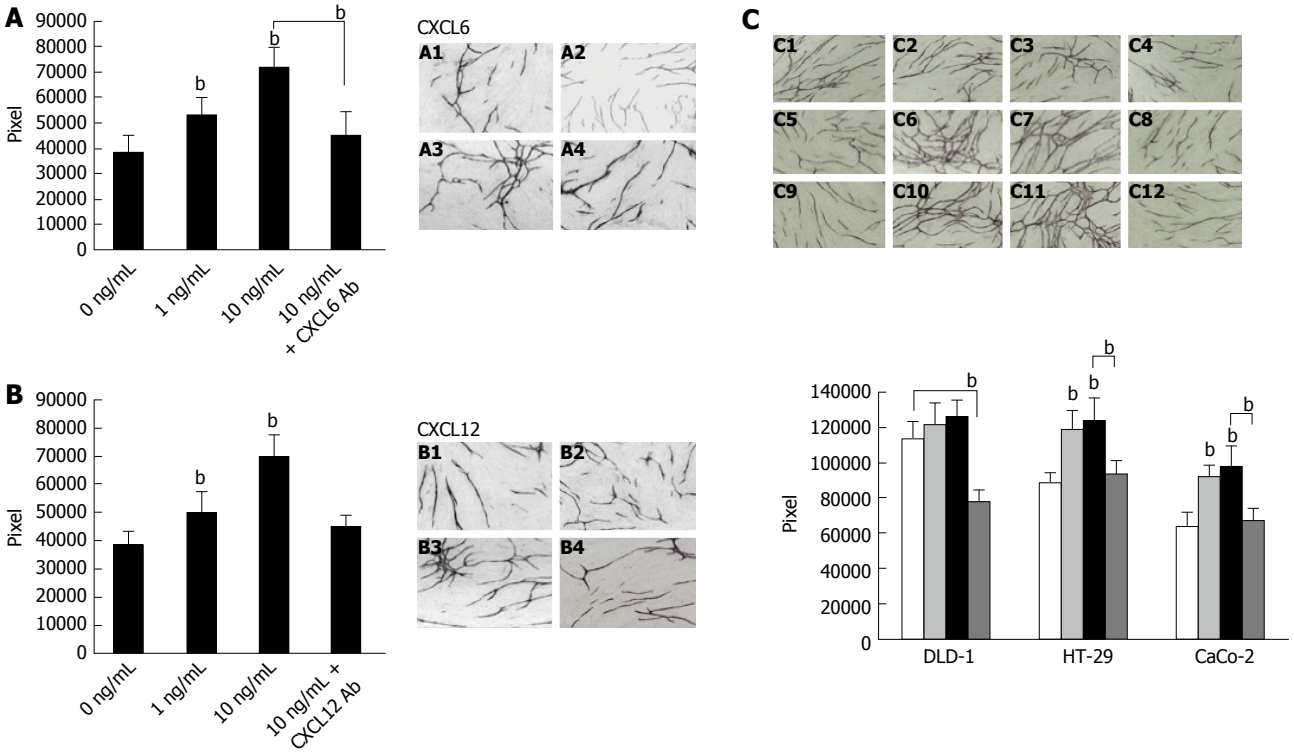

Figure 5 Effect of granulocyte chemotactic protein-2, stromal cell-derived factor-1 and co-culture with colon cancer cells on angiogenesis. The treatment of CXCL6 (A) and CXCL12 (B) influence HUVEC tube formation. After incubation of the HUVEC/fibroblast co-culture system in the presence or absence of CXCL6 or anti-CXCL12 Ab, then co-culture for 7 d, the HUVEC/fibroblast co-culture system was stained with anti-CD31 antibody. Tube formation area was measured quantitatively using an image analyzer. A1: Control; A2: 1 ng/mL CXCL6; A3: 10 ng/mL CXCL6; A4: 10 ng/mL CXCL6 + 10 μg/mL of CXCL6 Ab. B1: Control; B2: 1 ng/mL CXCL12; B3: 10 ng/mL CXCL12; B4: 10 ng/mL CXCL12 + 10 μg/mL of CXCL12 Ab. Effect of colon cancer cells (DLD-1, HT-29 or CaCo-2) on HUVEC tube formation is shown (C). Angiogenesis assay by HUVEC/fibroblast co-culture with DLD-1, HT-29 or CaCo-2 cells was conducted using the double-chamber method. Detection of tube formation by HUVECs was described in the "Material and Methods" section. C1: Co-culture with DLD-1; C2: Co-culture with DLD-1 + 10 ng/mL of CXCL6; C3: Co-culture with DLD-1 + 10 ng/mL of CXCL12; C4: Co-culture with DLD-1 + 10 μg/mL of CXCL12 Ab; C5: Co-culture with HT-29 cells; C6: Co-culture with HT-29 cells pre-treated with 10 ng/mL CXCL6; C7: Co-culture with HT-29 cells pre-treated with 10 ng/mL CXCL12; C8: Co-culture with HT-29 + 10 μg/mL of CXCL12 Ab; C9: Co-culture with CaCo-2 cells; C10: Co-culture with CaCo-2 cells pre-treated with 10 ng/mL CXCL6; C11: Co-culture with CaCo-2 cells pre-treated with 10 ng/mL CXCL12; C12: Co-culture with CaCo-2 cells pre-treated with 10 μg/mL anti-CXCL12 antibody. Columns, mean pixels of HUVEC tube formation area; Bars = SD. Multiple comparisons were performed by one-way ANOVA followed by the SNK test; $^{b}P < 0.01$ *vs* control. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

As a structural component of tumor tissue, fibroblasts have been shown to be deeply involved in tumor proliferation and the mitogenic processes. Fibroblasts produce certain cytokines that influence neighboring cells, including malignant cells[4]. The precise role of chemokines in neovascularization during inflammation or tumor growth is not yet fully understood. We investigated here whether cancer cell stromal cell-derived CXCL12 influences colon cancer CXCL6 secretion, thereby co-regulating the metastatic potential of colon cancer. Our results revealed that CXCL12 was expressed in DLD-1 and fibroblasts, while CXCL6 and CXCR4 were expressed in all cell lines.

The most salient observations of our study were that the secreted CXCL6 levels by colon cancer cells and HUVECs were significantly promoted by cancer cell (DLD-1)- and stromal cell (fibroblast)-derived CXCL12 in the co-culturing system, and that the enhanced CXCL6 production could be significantly inhibited by CXCL12 Ab. Similar results were reported for other effects through the up-regulation of MMP-9, providing a possible mechanism mediating the effect of CXCL6 on metastasis[34]. In our study, CXCL6 and CXCL12 not only co-operatively enhanced proliferation and invasion of HUVECs, but also promoted the invasion of colon cancer cells. Similarly, CXCL6 has been reported to be up-regulated in colon cancer, and plays key roles in the induction and maintenance of gut inflammation, enhancing the development and growth of colitis-associated colorectal cancer[35].

To further investigate the inaction between CXC chemokines and cancer cell living microenvironment, we focused on the interaction between tumor cells and stromal cells by characterizing angiogenic activity in co-cultured fibroblasts and vascular ECs, and the effect of CXCL6 and CXC12 in this system. HUVEC tube formation was significantly enhanced by CXCL6. We aimed to explore the influence of different secreted CXCL2 from colon cancer cells on tube formation by HUVECs. The tubular formation was significantly enhanced by co-culture with DLD-1 cells, as compared with colon cancer cells, and this is related to the produced CXCL12. In contrast, the enhanced tubular formation by HUVECs was significantly inhibited by

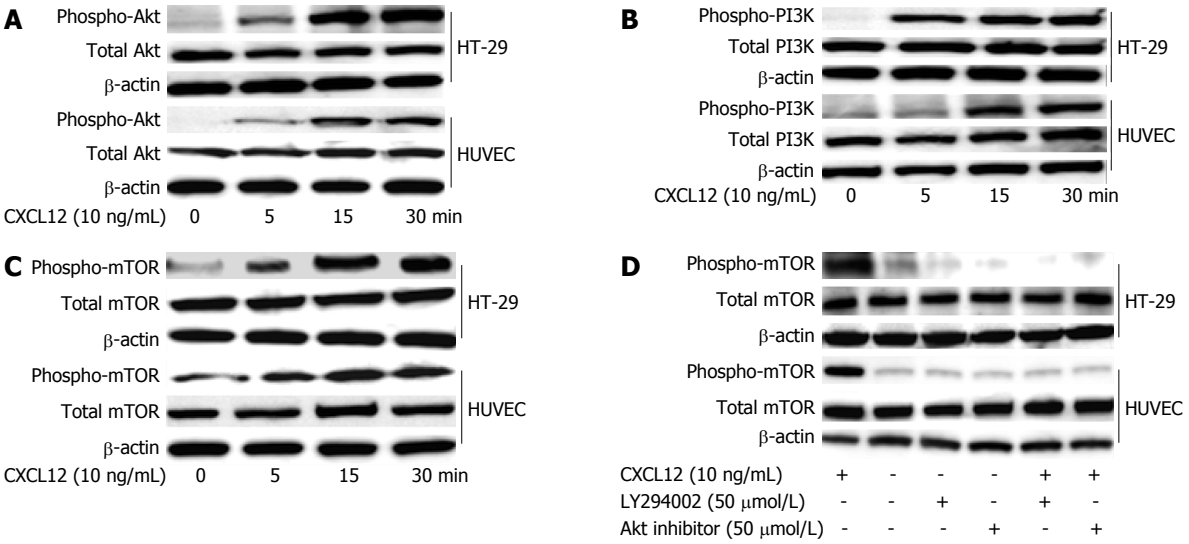

Figure 6 Stromal cell-derived factor-1-induced phosphorylation of PI3K/Akt/mTOR signaling in colon cancer cell lines and stromal cells. HT-29 cells and HUVECs were treated with 10 ng/mL of CXCL12 cultured for 5, 10 and 30 min. The cells were collected and lysed by lysis buffer. Aliquots of 30 μg of lysed protein were subjected to immunoblotting with a phospho-Akt (A), phospho-PI3K (B) and phosphor-mTOR (C) Abs. Detection of total Akt, PI3K or mTOR levels aided in loading control. HT-29 cells or HUVECs, after being pre-treated with 50 μmol/L Akt inhibitor and 50 μmol/L LY294002 for 1 h, were incubated with 10 ng/mL CXCL12 for 1 h. Results of immunoblotting using the mTOR Ab is shown (D). Detection of total mTOR levels served as loading control. Ab: Antibody; CXCL6: Granulocyte chemotactic protein-2; CXCL12: Stromal cell-derived factor-1; HUVEC: Human umbilical vein endothelial cell.

addition of anti-CXCL12 Ab in co-culture with DLD-1 cells (Figure 5). CXCL12 should be the initial factor secreted by fibroblasts, and the target colon cancer cells enhanced the secretion of CXCL6 after CXCL12 combined with its receptor CXCR4. The proliferation and invasion of colon cancer cells and HUVECs have been activated and enhanced after a series of complicated biochemical reactions.

Breakthroughs of insights into the tumor microenvironment have made great contributions towards clinical treatment. All kinds of anti-carcinoma chemotherapeutics have been based upon this mechanism, and there is no exception among the newly targeted cancer therapies or the gene therapies; proof of effects on critical pathways in proliferation or differentiation are sought. Chemokines are chemo-attractant cytokines that regulate genetic activity of leukocytes and other cell types, including tumor and stromal cells[36]. It is already known that mTOR is an atypical intracellular serine/threonine protein kinase regulated by PI3K. The activated mTOR pathway has been identified in several human malignancies, thus being an attractive target for anticancer therapy[37].

Our results showed that CXCL12-enhanced secretion level of CXCL6 co-regulation of invasion, proliferation and angiogenesis were dependent on PI3K-Akt-mTOR signaling activation. However, both of these factors up-regulation of PI3K-Akt-mTOR survival signaling were decreased by selective inhibitors of PI3K and Akt. All these results suggest that both the CXCL12 factor and the enhancement of CXCL6 expression serve to co-operatively promote metastatic potential in colon cancer cells. CXCL12-induced activation of this signaling pathway could be inhibited by PI3K/Akt inhibitor, consistent with the inhibition of metastatic capabilities of colon cancer cells. This cascade may be a key pathway for colon cancer cells to metastasize.

Crosstalk between CXCR4, CXCL12 and PI3K/mTOR has been previously described in peritoneal disseminated gastric cancer and pancreatic cancer. The solid tumors indicate an interconnection between CXCL12 and mTOR signaling. Interfering with mTOR signaling has abolished chemotaxis towards CXCL12[38]. The mTOR will enhance cell growth and proliferation *via* promotion of the ribosome S6 protein kinase (p70S6K) and inhibition of the eIF4E-binding proteins (4E-BP1), and can even enhance the secretion of vascular endothelial growth factor and angiogenesis by promoting expression of the transcription factor hypoxia-inducible factor 1 and its downstream target genes. Under a series of exterior and interior factors, cancer cell proliferation and invasion can be induced and cell apoptosis can be avoided by initiating the PI3K/Akt/mTOR pathway[39].

In conclusion, this is the first report on the concomitant involvement of CXC12 and CXCL6 both transducing the mTOR pathway, affecting progression and spreading of human colon cancer cells, ultimately suggesting that targeting of CXCR4 and mTOR may improve therapeutic efficacy and prevent mTOR-targeting agents' resistance. Our work should encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

COMMENTS

Background

Colon cancer is the fourth most frequently diagnosed cancer worldwide. The poor prognosis of colon cancer is attributable to its tendency of metastases.

However, the precise mechanisms of metastasis are still unknown. The target of this study was to investigate the underlying mechanism by which CXCL12 and CXCL6 influences the metastatic potential of colon cancer and the internal relation of colon cancer and stromal cell, as well as to investigate the interaction with CXCL12/CXCL6/PI3K/Akt/mTOR signaling in the metastatic process.

Research frontiers

The functions of CXC chemokines in the tumor microenvironment depend considerably on the chemokine type and tumor and stromal cell characteristics. Both the angiogenesis-promoting effect of CXCL6 and chemotactic effect of CXCL12 play important roles in tumorigenesis and metastasis. However, the molecular mechanisms of the activity signaling pathway by which CXCL12 and CXCL6 co-operatively regulate metastasis of colon cancer remain to be clarified.

Innovations and breakthroughs

This research provides the first demonstrations of fibroblast-derived CXCL12 enhancing CXCL6 secretion of colon cancer cells. CXCL6 and CXCL12 not only co-operative enhanced proliferation and invasion of HUVECs, but also promoted the invasion of colon cancer cells *via* the PI3K/Akt/mTOR signaling pathway. Blocking this pathway may be a potential anti-metastatic therapeutic target for patients with colon cancer. This work might encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

Applications

The concomitant involvement of CXC12 and CXCL6 transduces the mTOR pathway, affecting progression and spread of human colon cancer cells. The authors suggest that targeting CXCR4 and mTOR may improve therapeutic efficacy and prevent mTOR- targeting agents' resistance. The authors' work should encourage further investigation into more potent angiogenesis modulating agents to improve the effectiveness of colon cancer therapies.

Terminology

The CXC chemokine family of cytokines, found in the microenvironment, represent the significantly distinctive profiles of tumors and normal tissues. The tumor microenvironment contains secreted chemokines representing distinctive profiles, the components of each having specific target cells. The chemokine CXCL12, through its receptor CXCR4, positively regulates angiogenesis by promoting endothelial cell (EC) migration and tube formation.

Peer-review

The results of this study for the relationship between CXCL6 and CXCL12 in colorectal cancer and ECs seem to be of interest to many readers, and the experiment is well planned. But, before publication, several issues have to be considered.

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ORIGINAL ARTICLE

Case Control Study

Association between *CYP24A1* polymorphisms and the risk of colonic polyps and colon cancer in a Chinese population

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Author contributions: Chen XQ performed the majority of experiments and wrote the manuscript; Mao JY, Li WB and Li J participated in collection of the human material and clinical data; Yang H and Qian JM served as scientific advisors; Li JN designed the study, performed quality control of the data and algorithms, and reviewed and approved the final version of the manuscript.

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Institutional review board statement: All procedures performed in the studies involving human participants were carried out in accordance with the ethical standards of the institutional research committee of Peking Union Medical College Hospital, and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent statement: Informed consent was obtained from all participants included in the study.

Conflict-of-interest statement: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at lijn@pumch.cn.

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Abstract

AIM

To determine the pathogenesis and potential single nucleotide polymorphisms (SNPs) as screening sites for colonic polyps, colon cancer and ulcerative colitis, and to analyze the possible association between these genetic polymorphisms and the three diseases.

METHODS

We evaluated genetic polymorphisms in 144 newly diagnosed colonic polyp patients, 96 colon cancer patients and 44 ulcerative colitis patients. The four SNPs genotyped were rs4809957, rs6068816, rs6091822 and rs8124792. The control group consisted of 504 East Asians enrolled in the 1000 Genomes Project. Correlations between *CYP24A1* SNPs and the diseases were analyzed by Fisher's exact probability test.

RESULTS

CYP24A1 polymorphisms rs4809957 A/G and rs6068816 C/T showed a statistically significant association with risk of the three diseases, when both the genotypes and allele frequencies were considered. With regard to rs6091822 G/T, all three diseases were related to risk allele carriers (GT + TT) *vs* wild-type (GG), but the associations between the allele frequencies and the diseases were not significant. The risk of colonic polyps and colon cancer was related to the allele frequencies of rs8124792 G/A, and this association remained for genotype frequencies of this SNP.

CONCLUSION

Four SNPs are related to the risk of colonic polyps and colon cancer. G allele in rs6091822 G/T may play an anti-cancer role only if it is homozygous. The A allele, which is a minor component of rs8124792, may be indicated in the diagnosis of colonic polyps or colon cancer rather than ulcerative colitis.

Key words: *CYP24A1*; Single nucleotide polymorphisms; Colonic polyps; Colon cancer

Core tip: To determine the pathogenesis and potential single nucleotide polymorphisms (SNPs) as screening sites for colonic polyps and colon cancer, we examined four SNPs located in *CYP24A1* in patients with colonic polyps, colon cancer, ulcerative colitis and controls, and found a statistically significant association with risk of the three diseases. Our research represents the first investigation on *CYP24A1* gene polymorphisms in colonic polyp patients. These findings predicted a potential role of *CYP24A1* polymorphisms as biomarkers for population-level screening of colon cancer.

Chen XQ, Mao JY, Li WB, Li J, Yang H, Qian JM, Li JN. Association between *CYP24A1* polymorphisms and the risk of colonic polyps and colon cancer in a Chinese population. *World J Gastroenterol* 2017; 23(28): 5179-5186 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5179.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5179

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer incidence and mortality in China. It is the fifth most common tumor and the fifth leading cause of cancer-related deaths in China[1], and is predicted to increase in the future as the standardized incidence rate of CRC among Chinese people increased by 66.83% from 12.15/100000 in 1990 to 20.27/100000 in 2013[2]. To date, there are no ideal diagnostic tests, and fecal occult blood, flexible sigmoidoscopy and optical colonoscopy are the main screening methods for CRC in Europe, the United States and Asia[3-5]. The value of these screening methods in detecting early cancer and reducing CRC-related mortality is well established.

For population-based CRC screening in China, a two-step screening strategy has been recommended by the Chinese Center for Disease Control and Prevention, the Ministry of Health of China: the immunochemical Fecal Occult Blood Test (iFOBT) and a quantitative high-risk factor questionnaire as the primary screening test, with a full colonoscopy for follow-up screening[6]. However, only 37.2% of the target population accepted an iFOBT in a CRC screening program conducted in Hangzhou city[7]. The low participation rate in screening is also a common problem in other countries in Asia. The participation rate is mainly affected by insufficient staff, possible adverse events and differences in government insurance systems[8].

Thus, it is very important to identify novel molecular signatures as reliable biomarkers of CRC. Their application in a more advanced and easily obtainable primary screening test may improve CRC diagnosis in high-risk populations that require colonoscopy, and may be cost-effective. Albumin, haptoglobin, transferrin, pyruvate kinase (PK) isoenzyme type M2, calprotectin, Ca3 anaphylatoxin and colon-specific antigen (CCSA-3 and CCSA-4) have been reported as alternative biomarkers for the detection of CRC[3]. In addition, DNA-related markers have received considerable attention.

The association between CRC and vitamin D was observed in humans and confirmed in animal models and cell lines[9]. Interestingly, many cancers have been found to be associated with low serum level of the precursor 25-hydroxyvitamin D3 (25-D3), but not with serum concentration of the active vitamin D[10]. This may be due to the extra-renal autocrine/paracrine vitamin D system, which synthesizes and degrades the active 1,25-D3 (Vitamin D3, 1,25-dihydroxyvitamin D3, [1α,25-(OH)2D3]) locally. Thus, vitamin D hydroxylases play a prominent role in this process[11]. The *CYP24A1* gene encodes a vitamin D3 catabolic enzyme. The expression level of *CYP24A1* was found to be significantly higher in CRC tissues[12,13]. Although the mechanism of this up-regulation is unclear, *CYP24A1* may be an interesting candidate biomarker for use in the screening of colonic cancer.

It is estimated that 35% of CRC risk may be explained by heritable factors[14]. A single nucleotide polymorphism (SNP) is the most common genetic variation, and may be a reliable biomarker of the genetic background of patients to predict the risk of CRC[15]. The SNPs in *CYP24A1* gene have been partially determined. Pibiri *et al*[16] reported that rs6022990 was nominally associated with left-sided CRC (P = 0.018) in African Americans. Dong *et al*[17] found a statistically significant association between rs4809958 and colon cancer risk in patients from three states in the United States. However, the association between *CYP24A1*

Table 1 Characteristics of the study population

Variable	Cases			*P* value
	Colonic polyps, *n* = 144	Colon cancer, *n* = 96	Ulcerative colitis, *n* = 44	
Sex, *n* (%)				0.9631[1]
Males	70 (48.6)	48 (50.0)	21(47.7)	
Female	74 (51.4)	48 (50.0)	23 (52.3)	
Age in years, mean ± SD	56.1 ± 10.7	58.8 ± 14.1	55.0 ± 12.4	0.1401[2]

[1]*P* value was calculated by the χ^2 test; [2]*P* value was calculated by the ANOVA test.

gene polymorphisms and colonic polyps has never been determined. Given the crucial role of CYP24A1 in the development of cancer, it is plausible that the *CYP24A1* polymorphisms may affect the risk of colonic polyps and colon cancer.

To determine the pathogenesis and potential SNPs as screening sites for colonic polyps and colon cancer, we conducted a case-control study. In this study, we selected four SNPs located in *CYP24A1*, and examined these SNPs in patients with colonic polyps, colon cancer, ulcerative colitis and controls, analyzing the possible association between these genetic polymorphisms and the three diseases. To the best of our knowledge, this is the first investigation on *CYP24A1* gene polymorphisms in patients with colonic polyps, and the first investigation to study the relationship between SNPs in *CYP24A1* gene and colon cancer risk in a Chinese population.

MATERIALS AND METHODS

Study population

A total of 144 newly diagnosed colonic polyp patients, 96 colon cancer patients and 44 ulcerative colitis patients were enrolled in this study between January and May 2015. Eighty-three of the CRC cases were from Henan Cancer Hospital in Zhengzhou and the others were from Peking Union Medical College Hospital in Beijing, China. All patients had a confirmed diagnosis, fulfilling standard diagnostic criteria according to clinical, endoscopic, radiological and histopathological findings. All patients gave written informed consent. Five milliliter venous blood was collected from each patient to extract DNA, and all DNA samples and data were handled anonymously. DNA was isolated from peripheral blood leukocytes using the standard proteinase K digestion, phenol/chloroform extraction and ethanol precipitation, and stored at -80 ℃. Demographic and clinical data were collected during in-person interviews using a questionnaire, and included age, sex, ethnicity, residential region and date of diagnosis. The control group consisted of 504 East Asians enrolled in the 1000 Genomes Project, and included Han Chinese in Beijing, China (CHB), Japanese in Tokyo, Japan (JPT), Southern Han Chinese (CHS), Chinese Dai in Xishuangbanna, China (CDX) and Kinh in Ho Chi Minh City, Vietnam (KHV).

SNP selection and genotyping

The following four SNPs were genotyped: rs4809957, rs6068816, rs6091822 and rs8124792. All were single nucleotide substitutions, previously identified within the *CYP24A1* gene (www.ncbi.nlm.nih.gov/snp/). These SNPs have minor allele frequency (MAF) of ⩾ 5% in the Hap-Map CHB population. Genotyping was carried out using Sequenom MassARRAY assays and TYPER4.0 software (SEQUENOM Inc., San Diego, CA, United States). Primer sequences for PCR and single-base extension were designed by Assay Design 3.1 (SEQUENOM Inc.). Multiplex PCR was performed to amplify DNA isolated from the peripheral blood. PCR reactions were treated with shrimp alkaline phosphatase to neutralize unincorporated dNTPs. A single-base extension reaction was performed after PCR. Reactions were subjected to a 3-fold dilution with H_2O, and fragments were purified with resin, spotted onto Sequenom SpectroCHIP microarrays and scanned by MALDI-TOF mass spectrometry. The laboratory staff who conducted the genotyping assays was blinded to the patients' information. All reported *P* values were uncorrected unless stated otherwise.

Statistical analysis

Statistical analyses were carried out using the IBM SPSS Statistics 22.0 software (IBM Corp., Armonk, NY, United States). Correlations between *CYP24A1* SNPs and diseases were analyzed by Fisher's exact probability test. All tests were two-sided, and $P < 10^{-9}$ was considered statistically significant.

RESULTS

Population characteristics

A total of 144 incident cases of colonic polyps, 96 of colon cancer, 44 of ulcerative colitis and 504 controls were enrolled in this study. As shown in Table 1, the case groups and the control group had similar sex and age distributions.

Association analysis

Results of the analysis by genotype categories using Fisher's exact test are shown in Table 2. Table 3 shows the results of the analysis by alleles using the Chi-square test and odds ratio (OR) for the association of each polymorphism with the three diseases. The MAF and test for Hardy-Weinberg equilibrium in the controls for each SNP are shown in Table 3. All SNPs met quality-control measures for the Hardy-Weinberg equilibrium.

Rs4809957 A/G and rs6068816 C/T showed a statistically significant association with the risk of colonic polyps, colon cancer and ulcerative colitis,

Table 2 Association between the selected single nucleotide polymorphisms and risks of colon cancer, polyp and ulcerative colitis

Variable (MJ/MI)	Cases/controls	Genetic model	*P* for Fisher's test
rs4809957 A/G	Cancer/1KGeno	AG + GG *vs* AA	2.20E-16
		G *vs* A	2.20E-16
		GG *vs* AG + AA	2.20E-16
	Polyp/1KGeno	AG + GG *vs* AA	2.20E-16
		G *vs* A	2.20E-16
		GG *vs* AG + AA	2.20E-16
	UC/1KGeno	AG + GG *vs* AA	2.20E-16
		G *vs* A	2.20E-16
		GG *vs* AG + AA	9.37E-09
	Cancer/Polyp	AG + GG *vs* AA	0.7998
		G *vs* A	0.8036
		GG *vs* AG + AA	1.0000
	Cancer/UC	AG + GG *vs* AA	0.4362
		G *vs* A	0.4424
		GG *vs* AG + AA	1.0000
	Polyp/UC	AG + GG *vs* AA	0.3011
		G *vs* A	0.3096
		GG *vs* AG + AA	1.0000
rs6068816 C/T	Cancer/1KGeno	CT + TT *vs* CC	2.20E-16
		T *vs* C	2.20E-16
		TT *vs* CT + CC	2.20E-16
	Polyp/1KGeno	CT + TT *vs* CC	2.20E-16
		T *vs* C	2.20E-16
		TT *vs* CT + CC	2.20E-16
	UC/1KGeno	CT + TT *vs* CC	2.29E-09
		T *vs* C	2.20E-16
		TT *vs* CT + CC	2.20E-16
	Cancer/Polyp	CT + TT *vs* CC	1.0000
		T *vs* C	0.4635
		TT *vs* CT + CC	0.4546
	Cancer/UC	CT + TT *vs* CC	1.0000
		T *vs* C	0.2786
		TT *vs* CT + CC	0.2658
	Polyp/UC	CT + TT *vs* CC	1.0000
		T *vs* C	0.06703
		TT *vs* CT + CC	0.06164
rs6091822 G/T	Cancer/1KGeno	GT + TT *vs* GG	2.20E-16
		T *vs* G	1.06E-08
		TT *vs* GT + GG	0.000472
	Polyp/1KGeno	GT + TT *vs* GG	2.20E-16
		T *vs* G	4.06E-12
		TT *vs* GT + GG	0.001364
	UC/1KGeno	GT + TT *vs* GG	1.32E-13
		T *vs* G	2.40E-05
		TT *vs* GT + GG	0.2411
	Cancer/Polyp	GT + TT *vs* GG	1.0000
		T *vs* G	0.9258
		TT *vs* GT + GG	0.5180
	Cancer/UC	GT + TT *vs* GG	1.0000
		T *vs* G	0.8971
		TT *vs* GT + GG	0.3094
	Polyp/UC	GT + TT *vs* GG	1.0000
		T *vs* G	1.0000
		TT *vs* GT + GG	0.5456
rs8124792 G/A	Cancer/1KGeno	GA + AA *vs* GG	2.20E-16
		A *vs* G	2.20E-16
		AA *vs* GA + GG	0.000758
	Polyp/1KGeno	GA + AA *vs* GG	2.20E-16
		A *vs* G	2.20E-16
		AA *vs* GA + GG	4.75E-05
	UC/1KGeno	GA + AA *vs* GG	4.90E-09
		A *vs* G	2.91E-09
		AA *vs* GA + GG	0.06359
	Cancer/Polyp	GA + AA *vs* GG	1.0000
		A *vs* G	1.0000
		AA *vs* GA + GG	1.0000
	Cancer/UC	GA + AA *vs* GG	1.0000
		A *vs* G	1.0000
		AA *vs* GA + GG	1.0000
	Polyp/UC	GA + AA *vs* GG	1.0000
		A *vs* G	1.0000
		AA *vs* GA + GG	1.0000

1KGeno: Control from 1000 Genomes Project; Cancer: Colon cancer cohort; MI: Minor allele (*i.e.*, less common in controls); MJ: Major allele (*i.e.*, more common in controls); Polyp: Colonic polyp cohort; UC: Ulcerative colitis cohort.

when both the genotypes and allele frequencies were considered. The minimum OR for rs4809957 G when compared with A in ulcerative colitis patients was 0.008, 95%CI: 0.001-0.055, *P* = 1.5659E-26. ORs for rs6068816 C *vs* T in all diseases were high (OR = 32.086, 95%CI: 16.238-63.403 for colon cancer; OR = 48.918, 95%CI: 24.888-96.150 for colonic polyps; and OR = 18.260, 95%CI: 8.350-39.932 for ulcerative colitis). For rs6091822, all three diseases were related to minor allele carriers (GT + TT) *vs* major allele homozygotes (GG), but other types of associations (T *vs* G and TT *vs* GT + GG) were not significant. The frequencies and distributions of the genotypes and ORs for these associations are shown in Table 4. Risks of colonic polyps and colon cancer were both related to allele frequencies of rs8124792 G/A, and this association remained for genotype frequencies for this SNP. In ulcerative colitis patients, the difference in the distribution was not significant.

DISCUSSION

The function of vitamin D is traditionally recognized in calcium and phosphate homeostasis. However, the protective role of vitamin D against various cancers has been highlighted in recent research. The association between CRC and reduced serum vitamin D3 levels has been widely observed[9]. Vitamin D exerts its biological functions in its active form, vitamin D3. Vitamin D3 binds the nuclear vitamin D receptor (VDR), and then regulates hundreds of genes. Therefore, vitamin D3 has an influence on cell proliferation, differentiation, apoptosis, DNA repair mechanisms, inflammation and immune function[9]. It is confusing that the serum concentration of the active 1,25-D3 does not show a constant relationship with CRC, but low serum level of the precursor 25-D3 does[10]. The *in situ* autocrine/paracrine vitamin D system in colon cells or colon cancer cells may be an important contributor in the onset and progression of colon cancer, rather than the serum level of vitamin D3 which is mainly affected by the kidneys.

CYP24A1 encodes the enzyme 25-hydroxyvitamin D3 24-hydroxylase, a key enzyme that catabolizes 1,25(OH)2D3 to the less active form 25-D3, which is considered the main enzyme determining the biological

Table 3 Associations between the selected single nucleotide polymorphisms and colon cancer, colonic polyp and ulcerative colitis, and odds ratio for the association of each polymorphism with these diseases

Variable (MJ/Mi[a])	MAF[a]	HWE *P*[a]	Cases/controls	Genetic model	*P* for χ^2 test	Odds ratio for MI/MJ	95%CI	
rs4809957 A/G	0.393	0.476	Cancer/1KGeno	G *vs* A	2.50E-48	0.021	0.009	0.048
			Polyp/1KGeno	G *vs* A	1.64E-64	0.026	0.014	0.048
			UC/1KGeno	G *vs* A	1.57E-26	0.008	0.001	0.055
rs6068816 C/T	0.388	0.434	Cancer/1KGeno	T *vs* C	8.00E-47	32.086	16.238	63.403
			Polyp/1KGeno	T *vs* C	8.72E-68	48.918	24.888	96.150
			UC/1KGeno	T *vs* C	3.83E-22	18.260	8.350	39.932
rs6091822 G/T	0.284	0.208	Cancer/1KGeno	T *vs* G	3.72E-09	2.524	1.844	3.457
			Polyp/1KGeno	T *vs* G	1.37E-12	2.596	1.984	3.395
			UC/1KGeno	T *vs* G	0.00001	2.645	1.696	4.125
rs8124792 G/A	0.281	0.616	Cancer/1KGeno	G *vs* A	1.26E-13	0.083	0.036	0.188
			Polyp/1KGeno	G *vs* A	2.93E-18	0.086	0.044	0.170
			UC/1KGeno	G *vs* A	2.58E-07	0.062	0.015	0.256

[a]MAF and HWE were calculated among controls only. 1KGeno: Control from 1000 Genomes Project; Cancer: Colon cancer cohort; MI: Minor allele (*i.e.*, less common in controls); MJ: Major allele (*i.e.*, more common in controls); Polyp: Colonic polyp cohort; UC: Ulcerative colitis cohort.

Table 4 Frequencies and distributions of rs6091822 G/T and odds ratio for the association

Variable (MJ/Mi)		Cases			Controls			*P* for χ^2 test	Odds ratio, GT + TT *vs* GG	95%CI	
		GG	GT	TT	GG	GT	TT				
rs6091822 G/T	Cancer/1KGeno	0	142	2	262	198	44	1.30E-20	0.010	0.001	0.069
	Polyp/1KGeno	0	96	0				9.75E-29	0.006	0.001	0.046
	UC/1KGeno	0	42	1				1.53E-10	0.021	0.003	0.154

MJ: Major allele (*i.e.*, more common in controls); MI: Minor allele (*i.e.*, less common in controls).

half-life of vitamin D3[11]. One study found that the expression level of *CYP24A1* was aberrantly increased in CRC tissues both at the mRNA and protein levels compared with corresponding non-cancerous tissues from CRC patients[12], and another study revealed that the expression level of *CYP24A1* was absent or very low in normal colon mucosa[13].

The mechanism of this up-regulation is unclear. However, there are several hypotheses. *CYP24A1* expression is highly induced by 1,25-D3 in a VDR-retinoid X receptor-dependent manner, and a meta-analysis showed that very often VDR levels do not correlate with *CYP24A1*[11]. Approximately 50 different polymorphisms of *CYP24A1* have been identified, but are only partially characterized. None of the four selected SNPs in our study have previously been investigated in colon cancer patients.

In the present study, rs4809957 showed a statistically significant association with the risk of colonic polyps, colon cancer and ulcerative colitis, when both genotypes and allele frequencies were considered. The minimum OR for rs4809957 G when compared with A in ulcerative colitis patients was 0.008, 95%CI: 0.001-0.055, *P* = 1.5659E-26. Our results indicate that the G allele is a strong protective factor, especially for ulcerative colitis, while the ORs in colonic polyp and colon cancer patients were similar.

These findings are consistent with the distribution of this polymorphism in non-small cell lung cancer (NSCLC)[18]. Rs4809957, located in the 3′ untranslated region which is adjacent to the polyA microsatellite repeat, possibly affects the stability of *CYP24A1* mRNA. Rs4809957 has not been found to affect the function or structure of protein encoded by the enzyme. It is possible that the mechanisms protecting the colon from inflammation or carcinogenesis are different, but this requires further study.

Rs6068816 also showed a statistically significant association with the risk of these three diseases. ORs for rs6068816 C *vs* T in all diseases were high (OR = 32.086, 95%CI: 16.238-63.403 for colon cancer; OR = 48.918, 95%CI: 24.888-96.150 for colonic polyps; and OR = 18.260, 95%CI: 8.350-39.932 for ulcerative colitis). Thus, these findings indicate that rs6068816 T is a strong risk factor for colon cancer and colonic polyps. Inconsistently, the T of rs6068816 is a weak protective factor in NSCLC (TT *vs* CT + CC, adjusted OR = 0.40, 95%CI: 0.26-0.60)[18]. Changes in rs6068816 would not affect the amino acid sequence of the *CYP24A1* expression product, but may affect intron splicing.

For rs6091822, the risk of all three diseases was related to allele carriers (GT + TT) *vs* major allele homozygotes (GG), but other types of associations (T *vs* G and TT *vs* GT + GG) were not significant. The frequencies and distributions of rs6091822 G/T and the OR for the association are described in Table 4. As there were expected frequency numbers less than 5, the χ^2 test may not be sufficiently precise and Fisher's exact test showed that there appears

to be no difference in the distribution of TT in the different groups. Our results suggest that the G allele plays a novel anti-cancer role, especially when homozygous, and the presence of minor allele T, even in heterozygotes, can contribute to the presence of colonic polyps or even colon cancer. Rs6091822 has been reported to have a correlation with breast cancer, but this finding was not constant in a different cohort[19]. The biological behavior of rs6091822 deserves further investigation.

In our study, the risk of colonic polyps and colon cancer was related to the allele frequencies of rs8124792 G/A, and this association remained for genotype frequencies of this SNP. In ulcerative colitis patients, the difference in the distribution was not significant. The A allele of rs8124792 may indicate the diagnosis of colonic polyps or colon cancer rather than ulcerative colitis. However, this conclusion was derived from a small number of participants, and a large-scale study is needed to verify this finding.

Our findings supported the associations between SNPs on *CYP24A1* and the risk of colonic polyps and colon cancer, and predicted a potential role of *CYP24A1* polymorphisms as biomarkers for population-screening of colonic polyps and colon cancer. In China, a two-step screening method has been used. iFOBT and a questionnaire of high-risk factors are used in the first step. If the iFOBT is positive or the questionnaire reports high-risk factors, a colonoscopy is suggested as the second step. The addition of SNPs testing as primary screening may further decrease the number of high-risk subjects entering the second step and undergoing colonoscopy, thus reducing the medical cost and the rates of complications of colonoscopy. However, the sensitivity and specificity of SNPs tests deserve further investigation before it is applied in clinical practice.

Our research represents the first investigation on *CYP24A1* gene polymorphisms in colonic polyp patients. In our study, the polymorphisms had similar distributions to those in colon cancer. This is concordant with the onset and progression of colonic polyps and colon cancer. Most cancers without family aggregation and precancerous lesions in colon tissues are due to abnormal activation of the Wnt/β-catenin signaling pathway. 1,25-D_3 can down-regulate this signaling pathway in not only cancer tissues in CRC patients[20] but also in the non-malignant cell line LT97, which harbors an adenomatous polyposis coli mutation[21]. Few studies have focused on the associations between serum vitamin D and colorectal polyps, and different to the situation in CRC patients, one study found that serum vitamin D levels were not different between the colorectal polyp and control groups[22]. The role of SNPs in colonic polyps requires further study.

Inflammatory bowel disease (IBD) is significantly associated with having higher odds for vitamin D deficiency[23]. Several *in vivo* and *in vitro* studies have examined the role of vitamin D in immune-mediated diseases such as IBD[24,25]. The consequences of vitamin D deficiency on the gastrointestinal tract include, but are not limited to, decreased colonic bacterial clearance[24], reduced expression of tight junctions in the intestinal epithelium, and elevated T helper 1 cell-driven inflammation at the gut level[23]. However, it is unclear whether this is the result of IBD-related malabsorption due to intestinal mucosal damage, or whether it is a possible contributor to disease onset and progression. Several SNPs in the VDR gene appear to confer susceptibility to ulcerative colitis in Asians, but do not have a statistically significant effect on IBD risk in Europeans[26-28]. Our study demonstrated that SNPs in the vitamin D-related gene *CYP24A1* are associated with ulcerative colitis in Asians. Furthermore, this association is similar with that for colonic polyps and colon cancer. This suggests that SNPs participate in the onset or progression of ulcerative colitis, and are not only the result of ulcerative colitis-related malabsorption. Although the mechanism is unclear, it may be similar to the way in which vitamin D affects the risk of colonic polyps and colon cancer.

There are some limitations in the present study that must be considered. Firstly, although we present the results of several novel associations, we cannot rule out the possibility that some of these associations may be due to chance, or the possibility of genetic pleiotropy and linkage disequilibrium. Further trials with a larger study population are needed. Secondly, our findings cannot be generalized to the general population, as we included only patients from two hospitals in China as cases and East Asians as controls. Thirdly, we did not include cancer staging information and ulcerative colitis severity in our analysis, and inclusion of these factors may help to identify differences.

In conclusion, we evaluated the associations between rs4809957, rs6068816, rs6091822 and rs8124792, and the risk of colon cancer, colonic polyps and ulcerative colitis. We demonstrated that these four SNPs were related to colon cancer, colonic polyps and ulcerative colitis. In future studies, we will identify both population replication and functional validation to confirm our findings.

COMMENTS

Background

Colorectal cancer (CRC) is a leading cause of cancer incidence and mortality in China and it is very important to identify novel molecular signatures as reliable biomarkers of CRC. Given the crucial role of CYP24A1 in the development of cancer, it is plausible that the *CYP24A1* polymorphisms may affect the risk of colonic polyps and colon cancer and may be an interesting candidate.

Research frontiers

CYP24A1 polymorphisms have been partially determined, but the association between *CYP24A1* gene polymorphisms and colonic polyps has never been determined.

Innovations and breakthroughs

This research is the first investigation on *CYP24A1* gene polymorphism in

colonic polyp patients. In this study, the polymorphisms had similar distributions to those in colon cancer. This is concordant with the onset and progression of colonic polyps and colon cancer. At the same time, none of the four selected SNPs in our study have previously been investigated in colon cancer patients.

Applications

The addition of SNP testing as primary screening may further decrease the number of high-risk subjects entering the second step and undergoing colonoscopy, thus reducing the medical cost and the complications of colonoscopy.

Peer-review

The authors have investigated the association between *CYP24A1* polymorphisms and colon cancer, polyps and ulcerative colitis. They found some significant correlations on direct comparisons. The study is well conducted and expertly written.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical significance of glycemic parameters on venous thromboembolism risk prediction in gastrointestinal cancer

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Abstract

AIM

To investigate the possible predictive role of routinely used glycemic parameters for a first venous thromboembolism (VTE) episode in gastrointestinal (GI) cancer ambulatory patients - with or without clinically diagnosed type 2 diabetes (T2D) or obesity - treated with chemotherapy.

METHODS

Pre-treatment fasting blood glucose, insulin, glycated hemoglobin (HbA_{1c}) and homeostasis model of risk

assessment (HOMA) were retrospectively evaluated in a cohort study of 342 GI cancer patients. Surgery was performed in 142 (42%) patients with primary cancer, 30 (21%) and 112 (79%) of whom received neoadjuvant and adjuvant therapies, respectively. First-line chemotherapy was administered in 200 (58%) patients with metastatic disease. The study outcome was defined as the occurrence of a first symptomatic or asymptomatic VTE episode during active treatment.

RESULTS

Impaired glucose tolerance (IGT) or T2D were diagnosed in 30% of GI cancer patients, while overweight/obesity had an incidence of 41%. VTE occurred in 9.4% of patients (7% of non-diabetic non-obese), especially in those with a high ECOG score (P = 0.025). No significant association was found between VTE incidence and T2D, obesity, different tumor types, metastatic disease, Khorana class of risk, or different anti-cancer drugs, although VTE rates were substantially higher in patients receiving bevacizumab (17% *vs* 8%, P = 0.044). Conversely, all glucose metabolic indexes were associated with increased VTE risk at ROC analysis. Multivariate Cox proportional analyses confirmed that HOMA index (HR = 4.13, 95%CI: 1.63-10.5) or fasting blood glucose (HR = 3.56, 95%CI: 1.51-8.39) were independent predictors of VTE occurrence during chemotherapy.

CONCLUSION

The results here reported demonstrate that evaluating glucose metabolic asset may allow for VTE risk stratification in GI cancer, helping to identify chemotherapy-treated patients who might benefit from thromboprophylaxis. Further multicenter prospective studies involving a larger number of patients are presently needed.

Key words: Gastrointestinal cancer; Type 2 diabetes; Venous thromboembolism; Chemotherapy; Insulin resistance

Core tip: The predictive value of pre-treatment fasting blood glucose, insulin, HbA_{1c} and homeostasis model of risk assessment (HOMA) was investigated in a cohort of gastrointestinal (GI) cancer patients. Despite all investigated metabolic markers were associated with an increased VTE risk during chemotherapy at ROC analysis, only elevated HOMA index (HR = 4.13) or fasting blood glucose (HR = 3.56) had an independent predictive value in survival analyses after adjustment for major confounders. These results suggest that glycemic metabolic markers, mainly HOMA index, should be carefully monitored in chemotherapy-treated GI cancer patients, as they could help to identify patients who might benefit from thromboprophylaxis.

Guadagni F, Riondino S, Formica V, Del Monte G, Morelli AM, Lucchetti J, Spila A, D'Alessandro R, Della-Morte D, Ferroni P, Roselli M. Clinical significance of glycemic parameters on venous thromboembolism risk prediction in gastrointestinal cancer. *World J Gastroenterol* 2017; 23(28): 5187-5195 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5187.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5187

INTRODUCTION

Cancer patients are at increased risk for venous thromboembolism (VTE)[1]. Among the different subtypes of tumors, pancreas, stomach, or other gastrointestinal (GI) (*i.e.*, esophagus, liver, biliary) cancers have been reported to have the highest risk for incident VTE, whereas colorectal cancer is generally considered at low risk[2,3]. Furthermore, the individual risk of VTE in GI cancers could be boosted by active treatment, and the use of new biological drugs has led to a clinically relevant increase in thromboembolic complications, as in the case of bevacizumab in colorectal carcinoma[1,4].

Beside cancer- and treatment-related factors, VTE risk might be influenced by patient's individual factors and co-morbidities[1]. The possibility that co-morbidities, such as type 2 diabetes (T2D)[5-8] or obesity[9-11] may be linked to an increased risk of VTE has been raised in the general population, and VTE has been proposed as a marker of underlying cancer - especially of the GI tract - in patients with T2D[12]. However, data are often confusing and no consensus exists on the impact of these two co-morbid conditions on VTE incidence[13], especially in the oncology setting.

Independently of an association between clinically diagnosed T2D, or obesity, and VTE, elevated levels of fasting blood glucose, glycated hemoglobin (HbA_{1c}) or insulin resistance [IR, evaluated by the homeostasis model of risk assessment (HOMA)] have all been associated with an increased risk of VTE, either unprovoked[14-19] or cancer-associated[20]. The possibility of a causal link between hyperglycemia (and, thus, HbA_{1c} or the composite HOMA index) and VTE occurrence is biologically plausible and supported by the experimental finding that, in healthy non-diabetic subjects, increased blood glucose levels enhances blood coagulation[21]. However, the association between hyperglycemia (assessed according to HbA_{1c}) and VTE risk has been recently disproved[22].

It is worth noticing that all these evidences derived from studies performed in non-cancer patients. To the best of our knowledge, there are no data, so far, that specifically addressed this issue in oncologic patients, with the only exception of a study by our group demonstrating that breast cancer women with IR had an increased risk of chemotherapy-associated VTE - independently of T2D, or other related risk factors[20].

Table 1 Patients' characteristics *n* (%)

Characteristics	*P* value
Age (yr), mean ± SD (range)	65 ± 10 (30-85)
Gender	
Male	197 (58)
Female	145 (42)
Length of follow-up (mo), median (IQR)	11 (6-24)
Venous thromboembolism	
Pulmonary embolism	9 (2.6)
Deep venous thrombosis	22 (6.4)
Portal vein thrombosis	2 (0.6)
Port-a-Cath	1 (0.3)
Cumulative frequency	34 (9.9)
Khorana Class of risk	
Low	199 (58)
Intermediate	118 (35)
High	25 (7)
Site of primary	
Colon-rectum	237 (69)
Stomach	38 (11)
Pancreas	36 (11)
Biliary tract	16 (5)
Oesophagus	15 (4)
Stage of disease	
Primary	142 (42)
Metastatic	200 (58)
Performance status (ECOG)	
0	274 (80)
1	60 (18)
2	8 (2)
Body mass index, mean ± SD (range)	24.9 ± 4.0 (14.8-39.5)
Normoweight	200 (58)
Overweight	106 (31)
Obese	36 (11)
Type 2 diabetes	79 (23)
Impaired glucose tolerance	23 (7)

IQR: Interquartile range; ECOG: Eastern Cooperative Oncology Group.

IR, hyperglycemia and T2D are associated with several cancer types, other than breast, and accumulating evidence indicates that they could represent shared pathophysiological mechanisms in GI cancer and its co-morbidities. Accordingly, we hypothesized that, as in the case of breast cancer, a de-regulated glucose metabolism could be involved in GI cancer-associated VTE, as well. Therefore, the present study was designed to investigate the possible predictive role of routinely used glycemic parameters for a first VTE episode in GI cancer out-patients - with or without clinically diagnosed T2D - in whom chemotherapy might act as a thrombotic trigger.

MATERIALS AND METHODS

Patients and sample collection

Starting from January 2007, the PTV Bio.Ca.Re. (Policlinico Tor Vergata Biospecimen Cancer Repository) and the Interinstitutional Multidisciplinary Biobank of the IRCCS San Raffaele Pisana (SR-BioBIM, Rome, Italy) are actively involved in the recruitment of ambulatory patients with primary or relapsing/metastatic cancer, who are prospectively followed under the appropriate Institutional ethics approvals, as part of a Clinical Database and Biobank project. Among them, 342 patients with GI cancer completed the clinical assessment for VTE. Inclusion criteria for patients whose serum samples were stored in our Biobanks were: age above 18 years, to be at the start of a first chemotherapy regimen, an Eastern Cooperative Oncology Group performance status (ECOG-PS) $\leqslant$ 2 and adequate hematological, hepatic and renal functions. Exclusion criteria were: therapeutic doses of any heparin before enrolment or treatment with anticoagulant or anti-platelet drugs. No patient underwent surgery during follow-up, nor was admitted to clinic for an acute medical illness requiring thromboprophylaxis.

GI cancer was staged according to the TNM classification. Surgery was performed in 142 patients with primary cancer. The remaining 200 patients had metastatic disease and entered the study prior to the start of chemotherapy. Among the non-metastatic population, 30/142 (21%) and 112/142 (79%) patients received neoadjuvant and adjuvant therapies, respectively. First-line chemotherapy was instituted in all patients with metastatic disease. Details on anti-cancer drugs are summarized in Supplementary Table 1. Erythropoiesis stimulating agents (2.6%), granulocyte colony stimulating factor (3.5%) and steroids (17.6%) were used as supportive drugs. Patients' characteristics are summarized in Table 1.

Patients were regularly seen at scheduled visits; additional visits were arranged at the occurrence of clinically suspected VTE. Initial VTE risk stratification was performed by the Khorana Score (KS) at a $\geqslant$ 3-point cutoff, as per current recommendation[23]. All patients were followed up for a median period of 11 mo, during which outcomes were prospectively recorded. The study outcome was defined as the occurrence of a first symptomatic or asymptomatic VTE episode during active treatment. Deep vein thrombosis (DVT) was confirmed by venography or color-coded duplex sonography (in proximal DVT only). Pulmonary embolism (PE) was diagnosed by spiral computed tomography.

The study was performed in accordance with the principles embodied in the Declaration of Helsinki. All patients gave written informed consent, previously approved by our Institutional Review Boards.

Blood sampling and assessment of glycemic indexes

Fasting serum samples were obtained prior to chemotherapy from each recruited subject, aliquoted and stored at -80 ℃ in the facilities of the PTV Bio.Ca.Re. or of the SR-BioBIM. Routine chemistry studies, including fasting blood glucose (Hexokinase/Glucose-6-phosphate dehydrogenase-based methodology; Abbott Laboratories, Abbott Park, IL, United States), were performed on fresh samples within one hour from blood withdrawal on an ARCHITECT c8000 System (Abbott

Table 2 Glycemic indexes in gastrointestinal cancer patients - Comparison between patients who developed or not venous thromboembolism during chemotherapy

	Whole cohort	Venous thromboembolism		*P* value[1]
		Yes (*n* = 34)	No (*n* = 308)	
Glycemia (mg/dL), mean ± SD (range)	107 ± 38 (51-415)	122 ± 53 (60-339)	105 ± 36 (51-415)	0.019
Insulinemia (μIU/mL), median (IQR)	12.5 (8.8-20.1)	17.2 (11.9-24.5)	11.6 (8.7-18.6)	0.016
HOMA index, median (IQR)	3.0 (2.0-5.6)	4.8 (3.0-8.1)	2.8 (2.0-4.9)	0.006
HbA_{1c} (%), mean ± SD (range)	6.1 ± 0.8 (4.3-13.0)	6.2 ± 0.6 (5.2-8.0)	6.1 ± 0.8 (4.3-13.0)	0.436

[1]Student *t*-test or Mann-Whitney *U* test were used for normally distributed or non parametric variables, respectively. IQR: Interquartile range.

Laboratories). Fasting insulin levels were analyzed on serum samples using a fully automated Lumipulse G 600 II chemiluminescent enzyme immunoassay analyzer (Fujirebio Inc. Tokyo, Japan) according to the manufacturer's instructions. The HOMA index (a marker of insulin resistance) was retrospectively calculated for each participating subject from fasting blood glucose and insulin according to the formula: glucose (mg/dL) × insulin (μIU/mL)/405.

HbA_{1C} levels were immediately measured on EDTA anticoagulated whole blood by a Tosoh G7 Automated HPLC Analyzer - HbA1c Variant Analysis Mode (Tosoh Bioscience, Rivoli, TO, Italy), certified by the NGSP (National Glycohemoglobin Standardization Program) and traceable to the Diabetes Control and Complications Trial.

All measurements were ascertained while blinded to the sample origin and to the study endpoint.

Statistical analysis

Sample size of the study was based on the agreement to inclusion criteria and willingness to provide informed consent rather than on sample size calculations. However, estimation was later performed and showed that the recruited population was capable of yielding a power > 90%, at a two-sided 5% significance level. This was based on the assumption of a true HR of at least 2 (based on previous data on breast cancer)[20], an accrual period of no less than 2 years, an elapsed time between cycles within 30 d, and a median time-to-event of 2.5 mo.

Data are presented as percentages, mean ± SD, or median and interquartile range (IQR). Differences between percentages were assessed by χ^2 test. Appropriate parametric or nonparametric tests were employed for group comparison. The cut-off values were generated from continuous data by receiver operating characteristic (ROC) curve analyses performed by MedCalc Statistical Software version 13.1.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014). Bayesian analysis was performed, and positive (+LR) and negative (-LR) likelihood ratios were used to estimate the probability of having or not VTE. Cox proportional hazards analysis was performed with a free web-based application (http://statpages.org/). Survival curves were calculated by the Kaplan-Meier and log-rank methods using a computer software package (Statistica 8.0, StatSoft Inc., Tulsa, OK, United States). VTE-free survival time was calculated from the date of enrolment until the date of any VTE occurrence, or of the last follow-up. For administrative censoring, follow-up was ended on September 30th, 2016. For patients receiving neoadjuvant chemotherapy follow-up was terminated at completion of an entire antiblastic treatment and before surgery. All tests were two-tailed and only *P* values lower than 0.05 were regarded as statistically significant.

RESULTS

A condition of impaired glucose tolerance (IGT)/T2D was diagnosed in 30% of patients, while overweight (*n* = 105) or obesity (*n* = 35) was observed in 41% of cases. It is well recognized that overweight/obesity increases the chances of developing T2D; accordingly, the rate of T2D rose from 19% in normoweight to 27% and 36% in overweight and obese patients, respectively (*P* = 0.122). The distribution of glucose metabolic indexes is reported in Table 2. As shown, median pre-chemotherapy HOMA index was 3.0 in the overall GI cancer cohort (IQR: 2.0-5.6). As expected, median HOMA index was associated with BMI and increased steadily from 2.5 in normoweight to 3.8 and 3.9 in overweight and obese patients, respectively (Kruskal-Wallis H = 18.8, *P* = 0.0001). Similarly, IR was associated with the presence of IGT (median HOMA index 4.3) or T2D (median HOMA index 6.7).

VTE occurred in 9.9% (Table 1) of GI cancer patients (median TTE: 3.2 mo) (7.5% of non-diabetic non-obese patients), in agreement with previous reports[1]. In particular, 15 (3 non fatal sub-segmental PE, 2 portal vein thrombosis and 10 DVT) patients were incidentally diagnosed with asymptomatic VTE at time of CT-scan for restaging. Symptomatic VTE was diagnosed in the remaining 19 patients. Clinical characteristics of all 34 patients with VTE are reported in Supplementary Table 2.

Of interest, only 2 (8%) VTE events were recorded among the 25 classified as high-risk for VTE, as per current guidelines[23]. Conversely, VTE occurred in 10% and 11% of patients classified as low- or intermediate-risk according to Khorana[23], respectively. VTE incidence increased with high ECOG-PS (*P* = 0.025), but no significant difference was observed for VTE rates

Table 3 Receiver operating characteristics and Bayesian analysis of venous thromboembolism predictive value of glycemic parameters

	Fasting blood glucose	Fasting insulin	HOMA	HbA_{1c}
AUC (SE)	0.636 (0.06)	0.630 (0.05)	0.647 (0.05)	0.574 (0.06)
95%CI	0.582-0.687	0.576-0.681	0.593-0.697	0.519-0.627
Criterion[1]	103 mg/dL	12 μIU/mL	2.6	6.0%
Sensitivity	75%	75%	81%	59%
Specificity	65%	51%	48%	63%
PPV	18%	14%	14%	14%
NPV	96%	95%	96%	94%
+LR (CI)	2.12 (1.54-2.57)	1.54 (1.13-1.85)	1.56 (1.19-1.81)	1.60 (1.07-2.13)
-LR (CI)	0.39 (0.19-0.68)	0.49 (0.23-0.87)	0. 39 (0.16-0.78)	0.65 (0.38-0.96)
P value[2]	0.0135	0.0128	0.0045	0.1989

[1]Corresponding with highest Youden index; [2]Significance level P (Area = 0.5). AUC: Area under the curve; PPV: Positive predictive value; NPV: Negative predictive value; +LR: Positive likelihood ratio; -LR: Negative likelihood ratio.

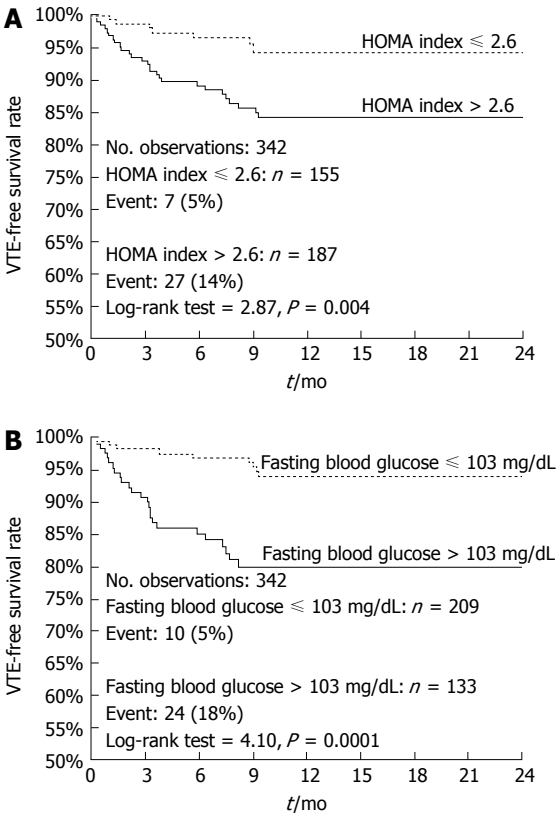

Figure 1 Kaplan-Meier curves of venous thromboembolism-free survival of the whole cohort of gastrointestinal cancer patients. Comparison between patients stratified on HOMA index (Panel A; HR = 3.17, 95%CI: 1.61-6.23) or fasting blood glucose (Panel B; HR = 4.21, 95%CI: 2.09-8.46).

among patients with colorectal (*n* = 25, 11%), gastro-esophageal (*n* = 5, 9.5%) or pancreatobiliary (*n* = 4, 7.7%) carcinomas. Similarly, no association was found with other clinical variables, such as metastatic disease, T2D, obesity, or different chemotherapy regimens, although VTE rates were higher in patients receiving bevacizumab (17% *vs* 8%, *P* = 0.044). On the other hand, pre-treatment fasting blood glucose, insulin, HOMA index, but not HbA_{1c}, were higher in patients who developed VTE during treatment compared with those who did not (Table 2).

ROC curves and bayesian analysis was, then, carried out to analyze the predictive performances of glycemic parameters. Details are reported in Table 3, showing that pre-treatment fasting blood glucose, insulin and the composite HOMA index, but to a lesser extent HbA_{1c} levels, were all associated with a significant probability of having or not VTE during chemotherapy. Hence, cut-off levels corresponding to the criteria associated with the highest Youden indexes were employed for patients' categorization and subsequent analyses. Multivariate Cox proportional analysis showed that, among all variables listed in Table 4, only ECOG-PS (HR = 2.47, 95%CI: 1.22-4.99) and HOMA index (HR = 4.13, 95%CI: 1.63-10.5) acted as independent VTE predictors (Overall Model Fit: Chi Square = 30.5, *P* = 0.0004). Of interest, when HOMA index was replaced by the individual parameters in an otherwise similar model of Cox analysis, only fasting blood glucose retained significance for VTE risk prediction (HR = 3.56, 95%CI: 1.51-8.39), along with ECOG-PS (HR = 2.34, 95%CI: 1.17-4.69) (Overall Model Fit: χ^2 = 36.9, *P* = 0.0001). Inclusion of anti-cancer drugs other than bevacizumab into multivariate models did not substantially modify the results obtained (data not shown).

Figure 1 demonstrates the Kaplan-Meier curves for VTE-free survival of GI cancer patients stratified on the basis of pre-treatment HOMA index (Figure 1A) or blood glucose levels (Figure 1B). As shown, patients with a HOMA index > 2.6 had a worse 1-year VTE-free survival rate compared to patients with a HOMA index below this cutoff (83% *vs* 94%, log-rank = 3.0, *P* = 0.003) (Figure 1A). Similarly, patients with glucose levels >103 mg/dL had a worse 1-year VTE-free survival rate compared to patients with glucose levels below this cutoff (78% *vs* 94%, log-rank = 4.5, *P* < 0.0001) (Figure 1B). These results were substantially confirmed in a sub-set of 261 non-obese non-diabetic GI cancer patients (Figure 2).

DISCUSSION

Diabetes and obesity have often been related with increased risk of VTE in the general population, but the

Table 4 Cox proportional hazards survival regression analysis of the predictive value of clinical-pathological variables and glycemic indexes on venous thromboembolism-free survival of gastrointestinal cancer patients *n* (%)

Variable	*n*	VTE		HR (CI)	*P* value
		Yes	No		
Sex					
Male	197	17 (9)	180 (91)		
Female	145	17 (12)	128 (88)	0.48 (0.24-0.97)	0.040
Age					
≤ 65 yr	157	9 (6)	148 (94)		
> 65 yr	185	25 (14)	160 (86)	2.15 (0.96-4.79)	0.062
Diabetes					
No	240	20 (8)	220 (92)		
IGT	23	2 (9)	21 (91)		
T2D	79	12 (15)	67 (85)	0.94 (0.62-1.41)	0.748
Tumor site					
Colorectal	237	25 (11)	212 (89)		
Stomach	38	3 (8)	35 (92)		
Esophagus	15	2 (13)	13 (87)		
Pancreas	36	3 (8)	33 (92)		
Biliary tract	16	1 (6)	15 (94)	0.78 (0.53-1.14)	0.201
Khorana class of risk					
Low	199	19 (10)	180 (90)		
Intermediate	118	13 (11)	105 (89)		
High	25	2 (8)	23 (92)	1.20 (0.64-2.26)	0.562
Stage of disease					
Primary	142	9 (6)	133 (94)		
Metastatic	200	25 (12)	175 (88)	1.81 (0.75-4.33)	0.186
ECOG-PS					
0	274	24 (9)	250 (91)		
1	60	7 (12)	53 (88)		
2	8	3 (37)	5 (62)	2.47 (1.22-4.99)	0.012
Homa index					
≤ 2.6%	155	7 (5)	148 (95)		
> 2.6%	187	27 (14)	160 (86)	4.13 (1.63-10.5)	0.003
Bevacizumab					
No	270	22 (8)	248 (92)		
Yes	72	12 (17)	60 (83)	1.77 (0.74-4.25)	0.199

ECOG-PS: Eastern Cooperative Oncology Group performance status; T2D: Type 2 diabetes; IGT: Impaired glucose tolerance; VTE: Venous thromboembolism.

evidences on their association in the oncology setting are sparse. Here we report, for the first time to our knowledge, that a condition of IR (assessed by the glucose/insulin composite HOMA index) associates to an increased risk of VTE in GI cancer out-patients on active chemotherapy. In particular, we demonstrate that a HOMA index > 2.6 at time of chemotherapy start is predictive for a first VTE event, independently of T2D, obesity or other well known risk factors, such as tumor site and stage or ECOG-PS. This correlation appears to be strongly dependent on blood glucose levels, rather than on insulin, as demonstrated by multivariate analyses in which glycemia, but not insulinemia, confirmed its independent association with VTE. Of note, HbA_{1c} - a marker of sustained hyperglycemia over the previous 2-3 mo - associated with VTE only in non diabetic GI cancer patients, with a HR of 3.12 (95%CI: 1.21-8.07, P = 0.02) independently of sex, age, BMI, IGT, ECOG-PS, and tumor site and stage, possibly as a result of the confounding effect of glycemic pharmacologic control in T2D (data not shown).

These results are scarcely comparable with literature data, as the majority of the studies investigated the predictive role of these metabolic features in the general population. Nonetheless, the finding of an independent predictive role of elevated fasting blood glucose levels in the GI cancer setting is in agreement with the results by Tala *et al*[18], demonstrating a 4.1 odds ratio of VTE in critically ill children with hyperglycemia. Similarly, Di Minno *et al*[14] indicated that impaired fasting glucose independently predicted idiopathic VTE in adults. Furthermore, the data here reported are partially in agreement with previously published results demonstrating that high HOMA scores were associated with an increased risk of VTE in the general population, which, however, was dependent on BMI[19]. This finding was further confirmed by Gariani *et al*[24], who suggested that the increased risk of diabetes-associated VTE might result from confounders (*i.e.*, obesity) rather than an intrinsic effect of diabetes. On the other hand, the predictive value of HOMA index in our analysis was independent of a condition of overweight/obesity, validating our previous results

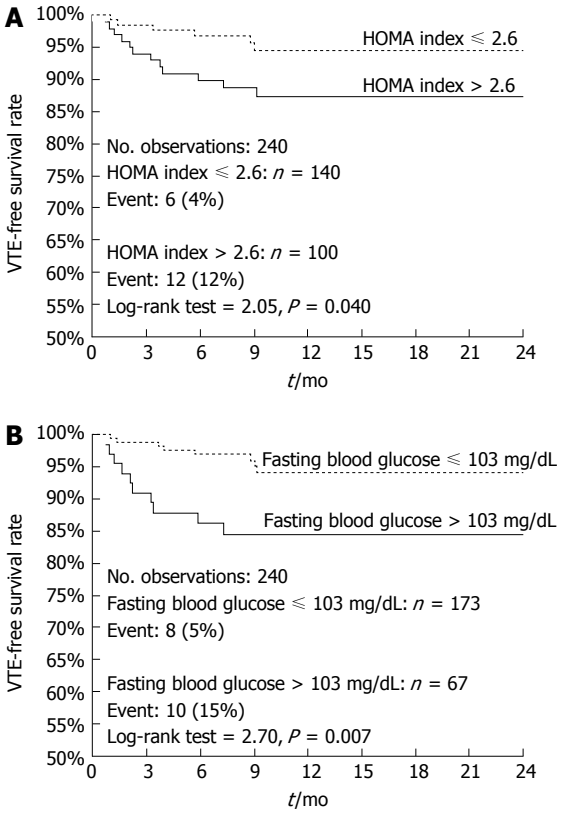

Figure 2 Kaplan-Meier curves of venous thromboembolism-free survival of non-diabetic non-obese gastrointestinal cancer patients. Comparison between patients stratified on HOMA index (Panel A; HR = 2.67, 95%CI: 1.05-6.80) or fasting blood glucose (Panel B; HR = 3.38, 95%CI: 1.19-9.57).

in a breast cancer cohort, in which a HOMA index greater than 2.5 (the cutoff for normality) predicted chemotherapy-associated VTE, independently of other components of the metabolic syndrome (BMI, T2D and HDL cholesterol)[20].

As stated above, the possibility of a causal link between hyperglycemia and VTE occurrence is biologically plausible and supported by the finding that increased blood glucose levels enhances blood coagulation in healthy non-diabetic individuals[21]. Additional evidence on the role of hyperglycemia on coagulation activation derives from experimental data suggesting that hyperglycemia may cause vessel damage through at least three apparently unrelated pathways: advanced glycation end product formation, activation of protein kinase C, and sorbitol accumulation by way of the polyol pathway (for a review see[25]). Furthermore, hyperglycemia *via* increased oxidative stress, and receptor for advanced glycation end products activation, increases the activation of transcription factor-κB in endothelial cells, thus causing a switch of the endothelial functions toward a pro-thrombotic, pro-inflammatory condition. This, together with an altered platelet metabolism and changes in intraplatelet signaling pathways, contributes to the pathogenesis of the thrombotic complications of T2D[25]. Of interest, in this study, elevated HbA_{1c} levels were significantly associated with increased mean platelet volumes (a marker of platelet hyperactivity available for 214 patients) either in the overall cohort (regression coefficient = 0.235, $P = 0.0006$) or in non-diabetic non-obese (regression coefficient = 0.239, $P = 0.004$) GI cancer patients, independently of age, sex or BMI (data not shown), thus suggesting a role for sustained hyperglycemia in the procoagulant status of these patients. However, this hypothesis is purely speculative, since the present study was not specifically designed to address this issue and, as such, it deserves further investigation.

Whichever the mechanism involved, cancer-associated VTE poses serious concerns both in terms of patient care and health costs, while thromboprophylaxis could provide an opportunity to substantially improve clinical management. Nonetheless, international consensus guidelines do not recommend routine prophylaxis for the primary prevention of VTE in cancer outpatients receiving chemotherapy, except for pancreatic cancer or selected high-risk patients[23,25,26]. To aid in VTE risk assessment, the use of Khorana score is currently recommended[23]. In the present study, the Khorana score was used to provide an initial estimate of VTE risk in GI cancer patients. However, it correctly identified VTE only in two gastric cancer patients (one normoweight diabetic and one overweight non diabetic) out of 25 patients classified as high-risk, both with IR. Conversely, 8.6% and 11% of patients classified as low- or intermediate-risk according to Khorana, had VTE. This is in agreement with recent findings suggesting that the major weakness of this scoring system is represented by the high proportion of the patients (> 50%) falling into the intermediate risk category[27], which also encompasses the majority of events[27-29]. At this point, it should be emphasized that, in the low- or intermediate-risk classes, 13 of 17 (77%) and 11 of 13 (85%) patients with VTE, respectively, had a HOMA index above the 2.6 cut-off. Thus, HOMA index, or other glucose metabolism parameters, could be employed in expanded risk scoring models, or newly developed clinical decision support systems[30] without causing an excessive increase in patients' management costs.

There are some limitations to acknowledge for this study. First of all, glucose metabolic parameters were retrospectively evaluated. However, all eligible consecutive patients within the designated timeframe were included and prospectively followed up, and all measurements were performed while blinded to the patient outcome. Secondly, recruitment was mono-institutional, and therefore might have limited external validity. Finally, analyses were conducted on a relatively small sample size, ultimately leading to a small number of events. Nonetheless, the results here reported suggest that a deregulation of glucose metabolism might contribute to VTE pathogenesis in chemotherapy-treated ambulatory cancer patients. To

the best of our knowledge, this is the first evidence reporting an association between HOMA index and VTE in GI cancer patients. At present, we may hypothesize that chemotherapy triggers a pro-thrombotic state, subdued in patients with hyperglycemia, independently of clinically overt T2D. Additional studies are required to validate this theory, as VTE can be influenced by many environmental or inherited factors that increase the likelihood of detecting spurious associations.

In conclusion, the results here reported demonstrate that the evaluation of glucose metabolic asset may allow for VTE risk stratification in GI cancer, helping to identify chemotherapy-treated patients who might benefit from thromboprophylaxis. Further multicenter prospective studies involving a larger number of patients are presently needed.

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COMMENTS

Background

The possibility of a causal link between hyperglycemia and venous thromboembolism (VTE) occurrence is supported by the experimental finding that, in healthy non-diabetic subjects, increasing blood glucose levels enhances blood coagulation. Accordingly, metabolic markers of impaired glucose metabolism have been associated with an increased risk of VTE in the general population, but little is known about their predictive significance in chemotherapy-treated cancer patients. The few available data come from a recent study demonstrating that breast cancer women with insulin resistance had an increased risk of chemotherapy-associated VTE, independently of type 2 diabetes (T2D), obesity, or other related risk factors.

Research frontiers

Future investigations specifically designed to address the predictive role of insulin resistance or hyperglycemia for VTE risk assessment may provide the rationale for their inclusion in expanded risk scoring models, or newly developed clinical decision support systems.

Innovations and breakthroughs

This study provides evidences that pre-treatment blood glucose levels or the composite homeostasis model of risk assessment (HOMA) index may allow for VTE risk stratification in GI cancer patients. Glycemic metabolic markers should be carefully monitored, independently of T2D or obesity, as they could provide important information for VTE risk prediction.

Applications

Clinicians should be alert to the pro-coagulant risk of impaired glycemic control and advise patients about lifestyle intervention, weight loss, and exercise as a part of their therapeutic plan. In the context of a precision medicine approach, incorporation of pre-treatment fasting glycemia - or HOMA index - in expanded risk scoring models, or newly developed clinical decision support systems might improve risk prediction without causing an excessive increase in patients' management costs.

Terminology

HbA_{1c} provides an estimate of average blood glucose levels over the previous three months and is used as a marker of glycemic control in the management of T2D. HOMA index, derived from blood glucose and insulin levels, is a non invasive parameter of insulin resistance in pathologic states, such as diabetes and obesity.

Peer-review

The article is interesting, methods of the study are presented concisely and there are no objections about it. The study deals with an important problem of treatment strategy in the large group of cancer patients, though the small number of subjects tends to show preliminary character of the study and does not allow to draw population conclusions.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Sex-dependent difference in the effect of metformin on colorectal cancer-specific mortality of diabetic colorectal cancer patients

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Data sharing statement: The original anonymous dataset is available on request from the corresponding author at taeilkim@yuhs.ac.

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Abstract

AIM

To assess factors associated with the higher effect of metformin on mortality in diabetic colorectal cancer (CRC) patients, since the factors related to the effectiveness of metformin have not been identified yet.

METHODS

Between January 2000 and December 2010, 413 patients diagnosed with both stage 3/4 CRC and diabetes mellitus were identified. Patients' demographics and clinical characteristics were analyzed. The effect of metformin on CRC-specific mortality and the interactions between metformin and each adjusted factor were evaluated.

RESULTS

Total follow-up duration was median 50 mo (range: 1-218 mo). There were 85 deaths (45.9%) and 72

CRC-specific deaths (38.9%) among 185 patients who used metformin, compared to 130 total deaths (57.0%) and 107 CRC-specific deaths (46.9%) among 228 patients who did not use metformin. In multivariate analysis, survival benefit associated with metformin administration was identified (HR = 0.985, 95%CI: 0.974-0.997, *P* = 0.012). Interaction test between metformin and sex after adjustment for relevant factors revealed that female CRC patients taking metformin exhibited a significantly lower CRC-specific mortality rate than male CRC patients taking metformin (HR = 0.369, 95%CI: 0.155-0.881, *P* = 0.025). Furthermore, subgroup analysis revealed significant differences in CRC-specific mortality between the metformin and non-metformin groups in female patients (HR = 0.501, 95%CI: 0.286-0.879, *P* = 0.013) but not male patients (HR = 0.848, 95%CI: 0.594-1.211, *P* = 0.365). There were no significant interactions between metformin and other adjusted factors on CRC-specific mortality.

CONCLUSION

We showed a strong sex-dependent difference in the effect of metformin on CRC-specific mortality in advanced stage CRC patients with diabetes.

Key words: Colorectal cancer; Metformin; Survival; Sex

Core tip: Evidence from previous studies has identified the anti-tumor effect of metformin; however, the factors related to effectiveness of metformin in diabetic colorectal cancer (CRC) patients have not been identified yet. Identifying subgroup patients who benefit from metformin treatment is important for future clinical application of metformin, and a strong sex-dependent difference of metformin effect in advanced CRC patients has been identified in this present study.

Park JW, Lee JH, Park YH, Park SJ, Cheon JH, Kim WH, Kim TI. Sex-dependent difference in the effect of metformin on colorectal cancer-specific mortality of diabetic colorectal cancer patients. *World J Gastroenterol* 2017; 23(28): 5196-5205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5196.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5196

INTRODUCTION

Although the survival rate for colorectal cancer (CRC) has increased because of early detection and intervention at earlier stages, CRC is still the 3rd most common cancer and the 4th leading cause of cancer death in the western and Asian countries[1-3]. Cancer and diabetes, especially type 2 diabetes mellitus (DM), are two of the most prevalent diseases and major causes of morbidity and mortality worldwide[4]. Despite some argument concerning the influence of diabetes on CRC, studies, including meta-analyses, have consistently demonstrated that type 2 DM is an independent risk factor for CRC and that diabetic patients with CRC have worse outcomes than non-diabetics[4,5]. A possible role for anti-hyperglycemic medications in progression and prognosis of CRC has been suggested, based on the hypothesis that tumor growth is promoted by the trophic action of insulin[4,6].

Metformin is broadly used for the treatment of type 2 DM, which successfully decreases circulating levels of glucose and insulin mainly by improving insulin resistance. Evidence from preclinical studies has identified the anti-tumor effect of metformin, showing inhibition of tumor growth and induction of apoptosis in cell lines and animal models of various cancers[7-9]. Several clinical studies, including our previous study[10-18], have shown the ability of metformin to reduce the incidence of CRC and improve survival of CRC patients. As stated in other studies, one of the potential mechanisms of the anti-tumor effect of metformin is *via* activation of AMP-activated protein kinase (AMPK). AMPK activation has an inhibitory effect on cancer cell growth and new blood vessel formation by prohibiting activation of the mammalian target of rapamycin (mTOR)[19-21]. With these direct cellular effects of metformin, the indirect or systemic effect of metformin is relief of insulin resistance-associated hyperinsulinemia and hyperglycemia, which counteracts the dependence of cancer cells on glucose as predominant source of energy[20,22].

Despite substantial evidence from *in vivo* and *in vitro* research supporting the possible efficacy of metformin as an anti-cancer agent and numerous clinical studies investigating the effect of metformin on CRC, particular factors or specific groups of patients associated with the effectiveness of metformin have not been identified. Our study assessed factors that may affect the efficacy of the anti-cancer action of metformin on CRC-specific mortality in diabetic CRC patients. Herein, we selected particular factors that might be associated with the "more effective" group (those who benefit from metformin for improving CRC-specific survival) and verified these assumptions using interaction analysis.

MATERIALS AND METHODS

Patients

The electronic records of 9472 consecutive patients with a diagnostic code of colon or rectal cancer seen at a single institution (Severance Hospital, Yonsei University, Seoul, Korea) between January 1, 2000 and December 31, 2010 were identified. A manual retrospective review was conducted for all patients to identify those with a prior history of DM. Among those identified, 1584 had the type 2 diabetes diagnostic

code during follow-up, of which 790 were excluded based on the following exclusion criteria: type Ⅰ diabetes (n = 38), diabetes diagnosed after CRC diagnosis (n = 521), incomplete records (including medication records) (n = 77), metformin use for less than 6 mo (n = 105), and any cancer previous to CRC diagnosis (n = 49). According to our previous studies, only stage 3 CRC patients[12] and resectable stage 4 CRC patients[23] showed a survival benefit from metformin. Considering these results, advanced stage CRC patients in the latter two groups, denoting stage 3 and 4 patients, were selected and analyzed; this group included 185 DM patients treated with metformin and 228 DM patients not taking metformin. There were 135 female patients (32.7%) in the study population.

Patient demographics and clinical characteristics, including age at diagnosis, sex, total follow-up duration, duration of diabetes, body mass index (BMI), family history of colorectal malignancy, smoking history and drinking history were obtained from medical records. BMI was stratified into "underweight" (BMI < 18.5), "normal" (BMI range: 18.5-24.9), "overweight" (BMI range: 25.0-29.9) and "obese" (BMI ⩾ 30.0), based on World Health Organization BMI classification[24]. Laboratory findings included plasma glucose levels, glycated hemoglobin (HbA1C) levels, and pretreatment carcinoembryonic antigen (CEA) levels. Information relevant to the CRC diagnosis, such as stage, site, histology, differentiation, resection margin, lymphovascular invasion, microsatellite instability (MSI) status, and treatment modality were reviewed *via* the medical records as well. The use of other diabetes medications (sulfonylureas, thiazolidinediones, α-glucosidase inhibitors, insulin, *etc.*) and the use of aspirin were also explored. The date of diagnosis of CRC was defined as the day of pathologic diagnosis. Every enrolled patient had undergone colonoscopy. We identified deaths through medical records, and determined the cause of death in all cases.

The institutional review board of Severance Hospital, Yonsei University, Seoul, Korea approved this study.

Tumor staging and treatment assessment

All patients were diagnosed with pathologically confirmed CRC and were evaluated during their baseline visit to Severance Hospital for appropriate staging according to the 7th version of the AJCC Tumor/Node/Metastatic staging system. Treatment modality was determined by extent and location of the tumor. Based on the National Comprehensive Cancer Network guideline, locally advanced tumors or advanced tumors with resectable metastatic lesions were treated by surgery followed by adjuvant chemotherapy with or without radiotherapy, or by neoadjuvant chemotherapy, or chemoradiation therapy followed by surgery. Advanced CRC with distant metastasis was treated by palliative chemotherapy or conservative care.

Statistical analysis

Differences between the metformin and non-metformin groups with regard to covariates were determined using Pearson's χ^2 test or Student's t-test when the data were categorical or continuous, respectively. In the primary analyses, the odds of overall and CRC-specific death for patients with diabetes treated with metformin and not treated with metformin were calculated using univariate logistic regression analysis. The multivariate Cox proportional hazards regression method was used to estimate HRs and 95%CIs after adjustment for patient-related variables, including age at diagnosis, sex, stage of cancer, BMI, diabetes duration, smoking history, cancer site, and use of insulin, aspirin, sulfonylurea and thiazolidinedione.

Survival curves were generated using the Kaplan-Meier method and were compared using log-rank statistics. In the secondary analyses, interaction analyses of Cox regression results were performed to reveal the factors associated with metformin use. These variables included age at diagnosis (⩾ 50 or < 50), sex (male or female), smoking history (yes or no), tumor stage (Ⅲ or Ⅳ), site (colon or rectum), sulfonylurea use (yes or no), insulin use (yes or no), and DM duration (years).

All P values were two sided, with a $P < 0.05$ considered significant. Most of the statistical analyses were performed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, United States). SAS version 9.2 (SAS Inc., Cary, NC, United States) was used when identifying the cut-off value of metformin duration that provided the best fit of the log-rank test statistics of overall and CRC-specific survival.

RESULTS

Patients' demographics and clinical characteristics

The metformin and non-metformin groups had similar patient demographics and clinical characteristics (Table 1). The median age of patients was 64 years (range: 33-91 years). Baseline characteristics, including age at diagnosis, sex, BMI, familial history of cancer, smoking and drinking history, were not significantly different between the metformin group and the non-metformin group. Factors associated with cancer, including tumor stage, tumor site (colon or rectum), tumor differentiation, resection margin positivity, MSI status, and pretreatment CEA level were not significantly different between the two groups. Clinical characteristics associated with diabetic severity status, such as HbA1C levels and duration of diabetes, were similar between the two groups; however, serum fasting glucose levels were lower in the non-metformin group compared to the metformin group (143.8 mg/dL *vs* 132.4 mg/dL, P = 0.012).

The use of other diabetes medications, including insulin, sulfonylurea and thiazolidinedione, was also investigated, because the status of individuals taking these medications could reflect later stage diabetes, and these medications could be associated with tumorigenesis and prognosis. The use of these

Table 1 Patient demographics and baseline clinical characteristics *n* (%)

	Metformin group, *n* = 185	Non-metformin group, *n* = 228	*P* value
Age at diagnosis in yr, mean ± SD	63.5 ± 8.789	63.49 ± 10.218	0.991
< 50	12 (6.5)	27 (11.8)	0.064
⩾ 50	173 (93.5)	201 (88.2)	
Sex			0.921
Male	125 (67.6)	153 (67.1)	
Female	60 (32.4)	75 (32.9)	
DM duration in yr, median (range)	8 (1-120)	6 (1-40)	0.068
Family history of CRC	8 (4.3)	13 (5.7)	0.526
BMI in kg/m^2, mean ± SD	23.6 ± 3.0	23.5 ± 3.0	0.596
Normal < 25	148 (80.0)	173 (76.5)	0.300
Overweight 25-30	33 (17.8)	51 (22.6)	
Obese ⩾ 30	4 (2.2)	2 (0.9)	
Smoking			0.371
Never-smoker	89 (48.1)	123 (53.9)	
Ex-smoker	42 (22.7)	40 (17.5)	
Current smoker	54 (29.2)	65 (28.5)	
Alcohol			0.556
None	82 (44.3)	112 (49.1)	
< 1 drink/d	42 (22.7)	51 (22.4)	
⩾ 1 drink/d	61 (33.0)	65 (28.5)	
Aspirin use	50 (27.0)	39 (17.1)	0.015
Insulin use	17 (9.2)	27 (16.2)	0.035
Sulfonylurea use	116 (62.7)	153 (67.1)	0.350
Thiazolidinedione use	18 (9.7)	12 (5.3)	0.082
CEA in ng/mL, median (range)	4.7 (0.2-9100.0)	6.4 (0.1-5946.0)	0.359
HbA1c, mean ± SD	8.7 ± 16.7	7.3 ± 1.4	0.349
Glucose in mg/dL, AC ± SD	143.8 ± 46.1	132.4 ± 41.1	0.012
Cholesterol in mg/dL, total ± SD	167.9 ± 47.8	164.6 ± 39.6	0.483
Tumor stage			0.110
Ⅲ	136 (73.5)	151 (66.2)	
Ⅳ	49 (26.5)	77 (33.8)	
Tumor site			0.940
Colon	106 (57.9)	130 (58.3)	
Rectum	77 (42.1)	93 (41.7)	
Histology			0.001
Adenocarcinoma	177 (97.8)	199 (89.6)	
Mucinous carcinoma	4 (2.2)	23 (10.4)	
Differentiation			0.155
Well differentiated	13 (7.4)	16 (7.8)	
Moderately differentiated	148 (84.6)	174 (84.5)	
Poorly differentiated	14 (8.0)	12 (5.8)	
Resection margin +	3 (1.7)	1 (0.6)	0.371
Lymphovascular invasion	56 (25.9)	76 (50.0)	0.031
MSI state			0.670
MSi	78 (89.7)	68 (90.7)	
MSI-low	6 (6.9)	6 (8.0)	
MSI-high	3 (3.4)	1 (1.3)	
Treatment modality			0.160
Resection only	9 (4.9)	20 (8.8)	
Resection + adjuvant chemotherapy	115 (63.2)	126 (55.3)	
Resection + chemoradiotherapy	22 (12.1)	24 (10.5)	
Neoadjuvant chemotherapy + resection	17 (9.3)	17 (7.5)	
Chemotherapy only	17 (9.3)	35 (15.4)	
Conservative care	2 (1.1)	6 (2.6)	

BMI: Body mass index; CEA: Carcinoembryonic antigen; CRC: Colorectal cancer; DM: Diabetes mellitus; MSI: Microsatellite instability.

medications was not significantly different between the two groups, with the exception of insulin use, which was lower in the metformin group than in the non-metformin group (9.2% *vs* 16.2%, *P* = 0.035). Aspirin, a drug known to have beneficial effects in cancer survival, was also evaluated and its use was statistically different between two groups (27.0% *vs* 17.1%, *P* = 0.015). Meanwhile, there was no difference in the treatment modality used for CRC between the two groups.

Metformin use and survival analysis

The median follow-up duration was 50 mo (range: 1-180 mo). With respect to the entire cohort, there were 129 (31.3%) recurrences, 215 (52.0%) total deaths, and 179 (43.3%) CRC-specific deaths. With

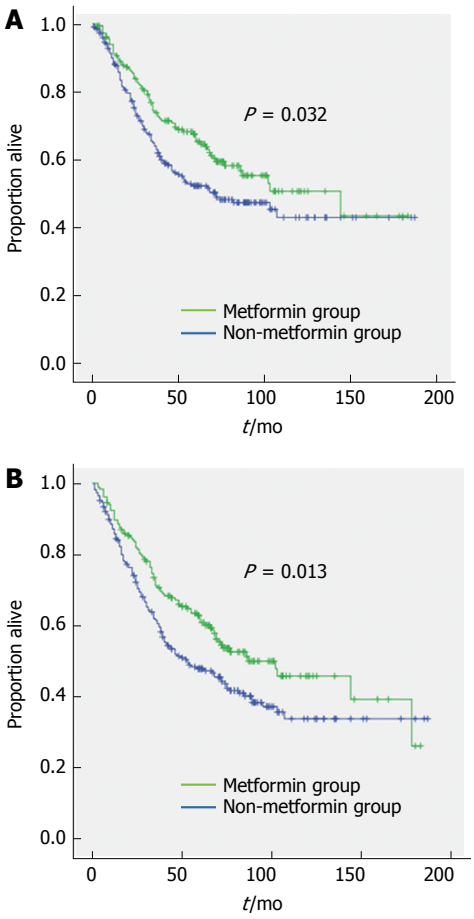

Figure 1 Colorectal cancer-specific survival and overall survival according to metformin treatment in colorectal cancer patients with diabetes mellitus. A: Colorectal cancer-specific survival according to metformin treatment; B: Overall survival according to metformin treatment.

respect to metformin use, there were 85 (45.9%) total deaths and 72 (38.9%) CRC-specific deaths among 185 patients who used metformin, compared with 130 (57.0%) total deaths and 107 (46.9%) CRC-specific deaths among 228 patients who did not use metformin. The estimated 5-year CRC-specific survival rates were 65.4% and 52.4% for the metformin and non-metformin groups, respectively, and 10-year CRC-specific survival rates were 50.8% and 43.1%, respectively. These results were significantly different (HR = 0.724, 95%CI: 0.537-0.976, *P* = 0.032) (Figure 1A). For the metformin and non-metformin groups, the estimated 5-year overall survival rates were 60.8% and 47.9% respectively, and the 10-year overall survival rates were 45.8% and 33.8% respectively, also showing significant differences (HR = 0.706, 95%CI: 0.537-0.929, *P* = 0.013) (Figure 1B).

In addition, we used the duration of metformin treatment in multivariate survival analysis, and showed this factor to be an independent predictor for CRC-specific mortality in diabetic patients with advanced CRC after adjustment of clinically relevant factors (HR = 0.985; 95%CI: 0.974-0.992, *P* = 0.012). BMI (HR = 0.514, 95%CI: 0.287-0.919, *P* = 0.025), tumor stage (HR = 8.401; 95%CI: 5.285-13.355, *P* < 0.001), and HbA1C level (HR = 1.015, 95%CI: 1.004-1.027, *P* = 0.01) were also revealed as independent predictive factors (Table 2).

Table 2 Multivariate logistic regression analysis for colorectal cancer-specific mortality

	HR	95%CI	*P* value
Age at diagnosis of ⩾ 50 or < 50	0.723	0.312-1.675	0.449
Sex, female or male	0.592	0.357-0.982	0.042
BMI of ⩾ 25 or < 25	0.514	0.287-0.919	0.025
Smoking history as yes or no	0.681	0.359-1.291	0.239
Aspirin use as yes or no	0.802	0.455-1.415	0.446
Metformin treatment duration in mo	0.985	0.974-0.997	0.012
Sulfonylurea use as yes or no	1.300	0.798-2.120	0.292
Insulin use as yes or no	1.041	0.511-2.121	0.912
Stage Ⅳ or Ⅲ	8.401	5.285-13.355	< 0.001
Site as rectum or colon	0.823	0.521-1.299	0.403
Pathology	0.801	0.238-2.701	0.721
Diabetes duration	0.968	0.935-1.001	0.059
HbA1C	1.015	1.004-1.027	0.010

BMI: Body mass index; CRC: Colorectal cancer.

We performed another analysis with the metformin group only, using total duration of metformin treatment. The results showed that improvement of CRC-specific (HR = 0.976, 95%CI: 0.948-0.995, *P* = 0.012) and overall survival rates (HR = 0.982, 95%CI: 0.967-0.997, *P* = 0.019) was associated with longer duration of metformin treatment, after adjustment of clinically relevant factors, including age at diagnosis, sex, medication history, tumor stage, tumor site, diabetes duration, and HbA1C. Analysis using Contal and O'Quigley's method[25] revealed that the cut-off value for metformin treatment duration that fit the CRC-specific survival statistics was 22 mo.

Interaction analysis of survival benefit from metformin treatment

To determine the subgroup with the greater metformin effect, interaction tests between metformin and each clinical factor were performed, after adjustment for other covariates including age at diagnosis, sex, BMI, medication use, stage, site, diabetes duration, and HbA1C. Interaction tests between metformin and sex with adjustment for relevant factors revealed that female CRC patients treated with metformin exhibited a significantly lower CRC-specific mortality rate compared to male CRC patients treated with metformin (HR = 0.369, 95%CI: 0.155-0.881, *P* = 0.025) (Table 3).

Subgroup analysis based on sex was performed and showed a significant difference in CRC-specific mortality between the metformin and non-metformin groups for females (HR = 0.013, 95%CI: 0.286-0.879, *P* = 0.013) (Figure 2B), while there was no significant

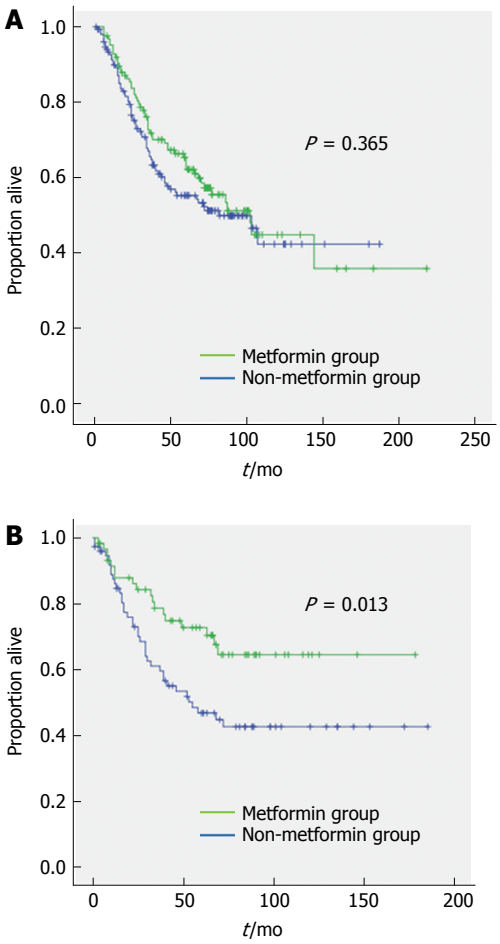

Figure 2 Colorectal cancer-specific survival based on sex. A: CRC-specific survival according to metformin treatment in males; B: CRC-specific survival according to metformin treatment in females. CRC: Colorectal cancer.

Table 3 Interaction analysis of metformin and relevant factors on colorectal cancer-specific mortality

	HR	95%CI	*P* value
Metformin-Sex	0.369	0.155-0.881	0.025
Metformin-BMI	1.000	0.974-1.026	0.972
Metformin-Site	0.941	0.441-2.006	0.875
Metformin-HbA1c	0.999	0.926-1.078	0.979

BMI: Body mass index.

difference between the two groups for males (HR = 0.365, 95%CI: 0.594-1.211, *P* = 0.365) (Figure 2A). Interaction analysis of metformin with other adjusted factors did not show any significant difference in CRC-specific mortality (Table 3). Intriguingly, as documented earlier, the duration of metformin treatment affected both CRC-specific mortality and overall mortality; however, the mean duration of metformin treatment between males and females was not significantly different (33.76 ± 24.45 mo for males and 28.05 ± 20.59 for females, log rank *P* = 1.06). Subgroup analysis based on metformin treatment showed that female patients had significantly lower CRC-specific mortality than males in the metformin group (HR = 0.332, 95%CI: 0.144-0.764, *P* = 0.009), while the non-metformin group showed no significant difference in CRC-specific mortality between male and female patients (HR = 0.73, 95%CI: 0.38-1.402, *P* = 0.345).

DISCUSSION

Two of the most common diseases worldwide, DM and CRC, share numerous risk factors. Previous studies, including meta-analyses, demonstrated the association between DM and increased risk of CRC; moreover, metformin, one of the most commonly prescribed anti-diabetes agents, improved survival of CRC patients[11,12,14,16,18]. We previously showed that CRC patients with diabetes treated with metformin had lower mortality than those not treated with metformin, and that metformin treatment was associated with a decreased incidence of colorectal adenomas in diabetic patients with previous CRC[12,26]. Furthermore, we showed an association between the metformin treatment in stage IV CRC patients with diabetes and lower risk of tumor recurrence after curative resection[23]. However, there has been no study that investigated the specific subgroup within CRC patients with diabetes who obtained a survival benefit from metformin use. In the present study, we aimed to determine the particular subgroup among diabetic CRC patients that could benefit from the anti-cancer effect of metformin and discovered that sex was the single clinical factor that predicted improved survival related to metformin treatment. In addition, by including the duration of metformin treatment as a factor, we showed that longer duration of metformin treatment was associated with improved CRC-specific and overall survival.

Metformin treatment has been associated with decreased risk and improved survival of DM patients with various types of cancer, including colorectal, pancreatic, liver, ovarian, breast, and endometrial[11,18]. The mechanism of action of metformin as an anti-cancer drug has not been clearly identified, although a shared pathogenesis for DM and some cancers is possible, *e.g.*, beta oxidation of fatty acids or mitochondrial function[22]. One of the most well-known mechanisms of metformin is the stimulation of peripheral AMPK with decreased hepatic gluconeogenesis, increased insulin sensitivity, and hepatic fatty acid oxidation[20]. Under physiological conditions, AMPK is an intracellular energy sensor and is activated when the cellular AMP/ATP ratio increases. AMPK activation leads to inhibition of mTOR signaling. mTOR phosphorylation is mainly involved in cell growth, cell cycle progression, and angiogenesis. Inhibition of mTOR signaling can be an excellent cancer therapy target, as the mTOR pathway is commonly decontrolled in numerous types of cancer, and activation of this

pathway is associated with poor prognosis and resistance to chemotherapy[21,27,28]. Other suggested anti-cancer mechanisms of metformin include reduced insulin growth factor-1, inhibition of angiogenesis, apoptosis, and induction of cell cycle arrest[6,29,30].

In our study population, females had a higher survival rate associated with metformin treatment after adjustment of other clinically significant factors. No other studies have reported the interaction between sex and survival benefit from metformin in diabetics with CRC. However, the study by Lee *et al*[18] of a cohort of 800000 Taiwanese showed that metformin effectively reduced the incidence of CRC in diabetic women and liver cancer in diabetic men, which suggested that sex could be an important interaction factor. Numerous explanations for this phenomenon can be suggested, and the higher survival rate of females compared to males among patients with CRC should initiate additional studies.

A Japanese study of 82402 patients with invasive CRC who had undergone surgery between 1985 and 2004 revealed a reduced risk of CRC-specific death for females relative to males that persisted over time[31]. McArdle *et al*[32] reported that overall survival and CRC-specific survival was significantly higher in females among patients who underwent elective surgery, after adjustment of clinical covariates. One study conducted in Israel by Purim *et al*[33] also reported sex-age interactions with the incidence of CRC and survival of CRC patients showed lower incidence and better prognosis for females. The answer for this superior CRC survival in females compared to males is usually related to female sex hormone status, particularly serum estrogen levels[31,34,35].

Circulating levels of 17b-estradiol (E2), the main estrogenic compound, are exceedingly higher in females compared to males and decrease with increasing age. While females are exposed to relatively high levels of endogenous E2 between adolescence and the 4th or 5th decade of life, in males, E2 levels remain low and steady, and drop minimally with aging. However, after menopause, serum E2 levels of females decline to levels similar to those of males. Moreover, the effect of estrogen on the gastrointestinal tract is well known, and in esophageal, gastric, and colon cancers, which have higher incidence and mortality rates among males, the role of estrogen has been investigated[36,37]. Wang *et al*[38] reported that people at risk of esophageal cancer have low levels of estrogen compared to healthy subjects. This finding was supported by experimental studies showing that estrogen regulates growth, cell differentiation, and cell function in the gastrointestinal tract. The possible role of estrogen in CRC development has been suggested by several lines of epidemiological, clinical and experimental evidence; however, the effect of estrogen in the progression of CRC has not been clearly identified[37,39].

With respect to metformin and female hormones, we hypothesized that metformin acts on the estrogen pathway to affect progression of CRC. This can be inferred from another result of Cossor *et al*[40], showing no significant survival benefit of metformin in post-menopausal females. Reports of the anti-cancer effect of metformin in estrogen receptor (ER)-positive breast cancer and the anti-estrogenic effect of metformin in control of abnormal endometrial proliferative disorders support this hypothesis[41,42]. The decrease in ER expression in tumors from females with endometrial cancer and DM treated with metformin compared to women treated with insulin also supports this hypothesis[42]. In addition, metformin repressed protein and mRNA expression of E2/ERα-regulated genes to a greater degree than tamoxifen, which resulted in inhibition of cell proliferation of ERα-positive breast cancer cells[41].

Interestingly, estrogen (E2) primarily prevented the development of CRC; however, in CRC patients, E2 promoted cancer progression[43]. Proliferation of CRC cells is known to be mediated by ERα, while the level of ERα expression is usually low in normal colon tissue and CRC tissue[43]. However, when the expression of ERβ in cancer cells decreases and the ratio of ERα/ERβ rises, ERα expression becomes dominant and results in cell proliferation and inhibition of apoptosis[43,44]. Interestingly, studies have demonstrated sex differences in ER expression in CRC[44,45]. Nüssler *et al*[44] reported a significant increase in ERα protein expression in males but not in females, while there was no significant difference in ERα and ERβ protein in normal colon mucosa between males and females. In the same study, ERβ protein expression in CRC cells was significantly decreased in both males and females, but far more in males[44].

Another study conducted by Press *et al*[45] reported the correlation between ERβ protein expression in CRC cells, overall survival and sex. Higher ERβ protein expression was associated with better overall survival in females but worse survival in males[45]. From these reports, we inferred that ER status in CRC tissue might have a role in cancer progression that could be different between males and females. The effect of metformin might be related to estrogen, regulation of ERα or ERβ expression, or, possibly, E2/ERα ratio as well. Although these relationships have not been elucidated thus far, we postulate that our findings provide the basis for future studies.

As confounding factors, DM severity and treatment with other drugs could affect the survival benefit conferred by metformin. Severity and duration of DM are important factors for cancer progression, considering that persistent hyperglycemia and hyperinsulinemia might alter the immune system and cause a chronic pro-inflammatory condition[4,5]. This pathologic state is due to the metabolic abnormalities that characterize diabetes, especially under conditions of poor metabolic control. In the present study, we

measured glycated hemoglobin to represent the severity of DM, and total duration since diagnosis of DM. In addition, other anti-hyperglycemic agents may conceal or diminish this metformin-related cancer protection.

Therefore, we adjusted DM severity and duration, along with other anti-hyperglycemic agents, to assess the dose-dependent survival benefit of metformin. Several studies have investigated the relationship between metformin duration or dosage-related numerical values and the incidence of CRC. While there are some discrepancies between the study results, one study showed that patients treated with metformin for over 3 years showed a significantly reduced relative risk of CRC (HR = 0.643, 95%CI: 0.490-0.845) compared to patients not treated with metformin[46]. Interestingly, Lee *et al*[18] demonstrated that total cancer incidence was significantly associated with mean daily dose of metformin. Furthermore, subgroup analysis of males and females showed other intriguing results; only the hazard ratio of liver cancer incidence was significantly associated with mean daily dose of metformin in males, while CRC incidence was significantly associated with mean daily dose in females[18]. Our study results showed the relationship between the cumulative effect of metformin and CRC-specific survival. In addition, duration of DM since diagnosis, duration of metformin treatment, and level of glycated hemoglobin were not significantly different between males and females, which showed that the severity of DM or months of metformin treatment had no effect on the sex-related interaction.

While this study provided notable associations between metformin treatment and sex in the survival of CRC patients with DM, there were some limitations. First, we could not capture metformin treatment non-compliance, which could have resulted in exposure misclassification and biased the results toward the null hypothesis. Additional study limitations included a small sample size, which reduced the power to detect significant differences in survival, even though our findings were similar to previous studies of metformin treatment and CRC outcomes. Data regarding the specific cancer location, such as right or left sided, were not available for this study. Location of cancer is an important difference between males and females, where females develop more proximal, and males more distal colon and rectal cancers[47,48]. Finally, because the data analyzed in this study population were collected from a tertiary medical care unit, results may not be generalizable to the general population. Further studies with a larger and more diverse population should be conducted to strengthen the relationship between sex and the anti-cancer effect of metformin in CRC patients with DM. Moreover, future prospective studies should consider this sex-specific difference when performing clinical trials using metformin as an additive therapeutic agent for diabetic and non-diabetic CRC patients.

COMMENTS

Background

Previous studies showed metformin use was associated with decreased colorectal cancer (CRC) mortality. The identification of factors associated with the effect of metformin on mortality in diabetic CRC patients will provide useful information when applying metformin in cancer treatment.

Research frontiers

Despite substantial evidence from *in vivo* and *in vitro* research supporting the possible efficacy of metformin as an anti-cancer agent and numerous clinical studies investigating the effect of metformin on CRC, particular factors or specific groups of patients associated with the effectiveness of metformin have not been identified. Herein, authors selected particular factors that might be associated with the "more effective" group, *i.e*., those who benefit from metformin for improving CRC-specific survival, and verified these assumptions using interaction analysis.

Innovations and breakthroughs

The authors discovered that sex was the single clinical factor that predicted improved survival related to metformin treatment. This is also the first study to report the interaction between sex and survival benefit from metformin in diabetics with CRC. Furthermore, results of this study showed the relationship between the cumulative effect of metformin and CRC-specific survival.

Applications

The results from this study showing sex-related effectiveness of metformin in survival of diabetic CRC patients can be applied to the additional usage of metformin in conventional adjuvant chemotherapy. These future prospective studies should consider this sex-specific difference when performing clinical trials using metformin as an additive therapeutic agent for diabetic and non-diabetic CRC patients.

Terminology

Metformin is an oral medication which is broadly used for the treatment of type 2 diabetes mellitus by decreasing circulating levels of glucose and insulin and mainly by improving insulin resistance. AMP-activated protein kinase and mammalian target of rapamycin are intracellular molecules associated with cell metabolism and growth.

Peer-review

This is a very good work, the authors addressed the factors associated with the effect of metformin on mortality in diabetic CRC patients. Interestingly, the results showed that female CRC patients taking metformin exhibited a significantly lower CRC-specific mortality rate than male CRC patients taking metformin. Identifying subgroup patients who benefit from metformin treatment is important for further study in this field and this manuscript provided interesting and valuable findings.

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ORIGINAL ARTICLE

Retrospective Study

Sex-influenced association of non-alcoholic fatty liver disease with colorectal adenomatous and hyperplastic polyps

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Author contributions: Chen QF, Zhou XD, Fang DH, Jin Y and Wu JS designed the study; Chen QF, Zhou XD, Sun YJ, Zhao Q and Huang JH collected the data; Chen QF and Zhou XD did the statistical analyses; Chen QF, Zhou XD, Fang DH, Jin Y and Wu JS reviewed the results, interpreted the data, and wrote the manuscript; all authors have made an intellectual contribution to the manuscript and approved the submission.

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Abstract

AIM

To investigate the relationship between non-alcoholic fatty liver disease (NAFLD) and colorectal adenomatous and hyperplastic polyps.

METHODS

A retrospective cross-sectional study was conducted on 3686 individuals undergoing health checkups (2430 males and 1256 females). All subjects underwent laboratory testing, abdominal ultrasonography, colonoscopy, and an interview to ascertain the baseline characteristics and general state of health. Multinomial logistic regression analysis was performed to examine the association between NAFLD and the prevalence of colorectal adenomatous and hyperplastic polyps.

Furthermore, the relationship was analyzed in different sex groups. Subgroup analysis was performed based on number, size, and location of colorectal polyps.

RESULTS

The prevalence of colorectal polyps was 38.8% in males (16.2% for adenomatous polyps and 9.8% for hyperplastic polyps) and 19.3% in females (8.4% for adenomatous polyps and 3.9% for hyperplastic polyps). When adjusting for confounding variables, NAFLD was significantly associated with the prevalence of adenomatous polyps (OR = 1.28, 95%CI: 1.05-1.51, $P < 0.05$) and hyperplastic polyps (OR = 1.35, 95%CI: 1.01-1.82, $P < 0.05$). However, upon analyzing adenomatous and hyperplastic polyps in different sex groups, the significant association remained in males (OR = 1.53, 95%CI: 1.18-2.00, $P < 0.05$; OR = 1.42, 95%CI: 1.04-1.95, $P < 0.05$) but not in females (OR = 0.44, 95%CI: 0.18-1.04, $P > 0.05$; OR = 1.18, 95%CI: 0.50-2.78, $P > 0.05$).

CONCLUSION

NAFLD is specifically associated with an increased risk of colorectal adenomatous and hyperplastic polyps in men. However, NAFLD may not be a significant factor in the prevalence of colorectal polyps in women.

Key words: Non-alcoholic fatty liver disease; Health-check; Adenomatous polyps; Hyperplastic polyps

Core tip: Non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of colorectal adenoma. The association of NAFLD and hyperplastic polyps remains unclear. We utilized 3686 individuals undergoing health checkups to analyze the relationship between NAFLD and colorectal adenomatous and hyperplastic polyps. NAFLD is associated with colorectal adenomatous and hyperplastic polyps in men but not in women.

Chen QF, Zhou XD, Sun YJ, Fang DH, Zhao Q, Huang JH, Jin Y, Wu JS. Sex-influenced association of non-alcoholic fatty liver disease with colorectal adenomatous and hyperplastic polyps. *World J Gastroenterol* 2017; 23(28): 5206-5215 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5206.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5206

INTRODUCTION

Colorectal adenomatous polyps and some subsets of hyperplastic polyps have been considered precursors of hereditary and sporadic colorectal cancer[1-5]. Currently, the incidence of colorectal polyps and colorectal cancer has been increasing over recent decades worldwide[6]. Identifying risk factors for colorectal cancer and performing colonoscopy and polypectomy are considered efficient surveillance programs to detect colorectal polyps and reduce malignant progression and mortality among the general population[7]. In a cross-sectional study of 343 patients who underwent colonoscopy, serum insulin levels as a metabolic factor were correlated with the prevalence of adenomatous (OR = 1.5, 95%CI: 1.1-2.0, $P = 0.005$) and hyperplastic polyps (OR = 1.3, 95%CI: 1.0-1.7, $P = 0.075$). In addition, some suggested that hyperplastic polyps could act as a marker for the future development of adenomas in the general population[8-11].

Non-alcoholic fatty liver disease (NAFLD), a metabolic disease of the liver, is well recognized as the most prevalent chronic liver disease worldwide, and its prevalence is dramatically increasing both in Asian and Western countries[12]. Patients with NAFLD present higher levels of insulin, insulin resistance status and chronic inflammation conditions[13-15]. Insulin and the insulin-like growth factor (IGF) axis play an important role in cell metabolism and growth. Insulin stimulates colorectal cell proliferation and increases bioactive IGF-1, which promotes cell cycle progression through mitogenic and antiapoptotic effects[16].

Recent studies suggested that NAFLD was associated with an increased risk for colorectal adenomas and advanced neoplasms[17-19]. Hwang *et al*[19] conducted an investigation of 2917 subjects divided into two groups (556 adenomatous polyps and 2361 polyp-free). They found that NAFLD was an independent risk factor for colorectal adenoma (OR = 1.28, 95%CI: 1.03-1.60, $P = 0.029$)[19]. However, this study ignored non-adenomas, such as hyperplastic polyps and inflammatory polyps, and investigated the association between NAFLD and non-adenomatous polyps. Limited data are available in the literature regarding the relationship between NAFLD and hyperplastic polyps. Given that insulin level was specifically associated with adenomatous and hyperplastic polyps and related to NAFLD, our aim was to explore the relationship between NAFLD and colorectal adenomatous and hyperplastic polyps.

In addition, Touzin *et al*[20] conducted a retrospective observational study to explore the relationship between biopsy-proven NAFLD and colorectal adenoma but failed to demonstrate that patients with biopsy-proven NAFLD had an increased incidence of colorectal adenoma. The association between NAFLD and colorectal polyps may be controversial. Furthermore, the prevalence of NAFLD (60.8% in males and 43.5% in females) and colorectal polyps (35.5% in males and 20.9% in females) was not consistent in different sex groups, and the prevalence is higher in males[21]. Several studies demonstrated a significant association between dyslipidemia and colon adenoma in men but not in women[22,23]. Therefore, our study further explored the link between NAFLD and colorectal adenomatous and hyperplastic polyps in different sex groups.

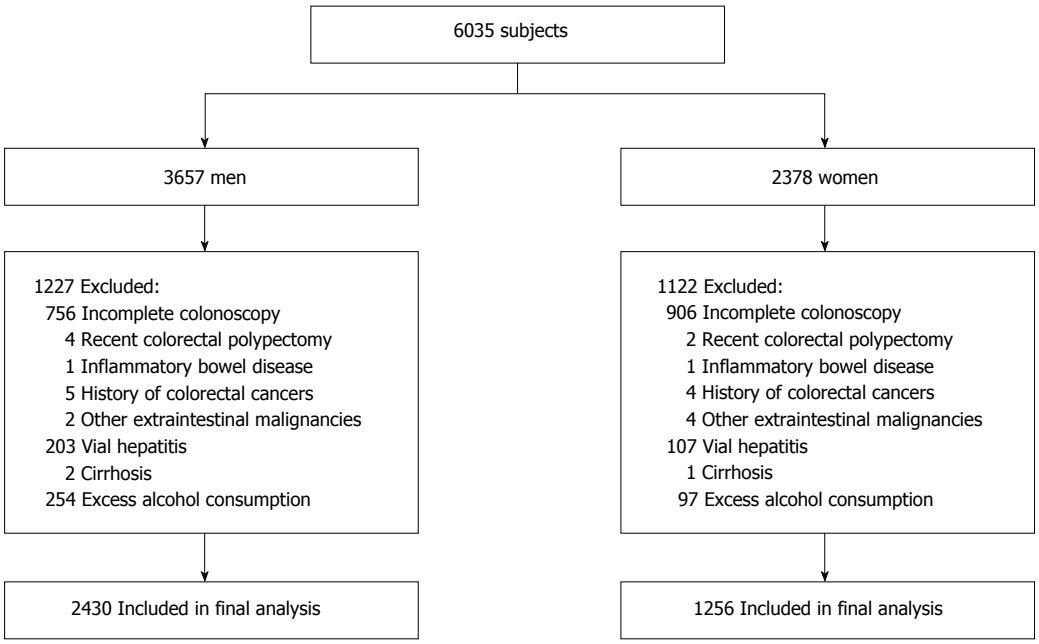

Figure 1 Inclusion and exclusion of study participants.

MATERIALS AND METHODS

Subjects

Study participants consisted of individuals who had a routine health checkup and colonoscopy at the Medical and Health Care Center of the First Affiliated Hospital of Wenzhou Medical University from September 2014 to November 2016. A standard questionnaire was administered to obtain relevant information from each participant, including smoking status (yes or no), alcohol consumption and medical history.

A total of 6035 participants were screened (Figure 1). In total, 2349 subjects were excluded based on the following reasons: incomplete colonoscopy, polypectomy, inflammatory bowel disease, carcinoma, liver cirrhosis, viral hepatitis, toxic and autoimmune liver diseases and excess alcohol consumption moderately (> 140 g/wk for men or > 70 g/wk for women). Thus, the final study population consisted of 3686 subjects (2430 males and 1256 females). Physical examination data were collected as part of a study, and formal ethical approval was not required[24].

Anthrometric index and laboratory tests

Well-trained examiners measured the body weight (measured to the nearest 0.1 kg) with a balance scale and the height (measured to the nearest 0.1 cm) with a portable stadiometer of every individual dressed in light clothes and without shoes in the morning. Body mass index (BMI) was calculated by dividing weight in kilograms by the height squared. Blood pressure was measured in the right arm in a seated state after they had at least 15 min of rest with a standard automatic instrument (Omron, model 705 cp, Kyoto, Japan) and recorded as systolic blood pressure (SBP) and diastolic blood pressure (DBP).

Following an 8-h fast, blood samples were collected and analyzed from antecubital vein sampling. Biochemical markers, such as fasting plasma glucose (FPG), triglyceride (TG), total cholesterol (TC), high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), alanine aminotransferase and aspartate aminotransferase, were subsequently analyzed with an automated analyzer (Abbott AxSYM, Park, IL).

Colonoscopy

Following full bowel preparation, colonoscopy (OLYMPUS, Tokyo, Japan) was performed in all participants by experienced gastroenterologists using a standard protocol identical for diagnostic colonoscopy[25]. Colonoscopic findings, including the type, location, size and number of polyps, were recorded. All polypectomy specimens were classified by experienced histopathologists. Based on the results of colorectal biopsies, individuals were divided into four groups: polyp-free group, adenomatous polyps group, hyperplastic polyps group and inflammatory polyps group. The location was stratified as following: (1) proximal lesions (polyps located in the cecum, ascending and transverse colon); (2) distal lesions (polyps located in the splenic flexure, descending sigmoid colon and rectum); and (3) bilateral lesions (polyps locating on both sides). The size of polyps was divided into two groups. Less than 10 mm was defined as small, whereas 10 mm or greater was defined as large. The number of polyps was also divided into two groups: single and multiple.

Diagnostic criteria

Abdominal ultrasonography was performed by professional radiologists to diagnose fatty liver. According

Table 1 Demographic and clinical characteristics of the subjects according to colonoscopic findings *n* (%)

Characteristic	Colonoscopic findings of 3686 subjects						
	Polyp-free	Colorectal polyps	*P* value *vs* Polyp-free	Adenomatous polyps	*P* value *vs* Polyp-free	Hyperplastic polyps	*P* value *vs* Polyp-free
	2501 (67.9)	1185 (32.1)		498 (13.5)		288 (7.8)	
Male	1487 (59.5)	943 (79.6)	< 0.001	393 (83.0)	< 0.001	239 (77.9)	< 0.001
Age (yr)	45.47 ± 9.99	50.48 ± 9.96	< 0.001	51.77 ± 9.78	< 0.001	48.16 ± 9.09	< 0.001
Weight (kg)	64.27 ± 11.29	68.33 ± 11.04	< 0.001	67.58 ± 11.13	< 0.001	69.57 ± 11.01	< 0.001
Height (cm)	164.64 ± 7.71	166.59 ± 7.26	< 0.001	165.95 ± 7.47	0.001	167.46 ± 7.01	< 0.001
BMI (kg/m^2)	23.62 ± 3.22	24.54 ± 3.10	< 0.001	24.45 ± 3.10	< 0.001	24.75 ± 3.13	< 0.001
SBP (mmHg)	124.43 ± 17.78	129.99 ± 18.34	< 0.001	130.57 ± 18.75	< 0.001	127.72 ± 17.94	0.003
DBP (mmHg)	73.48 ± 12.27	76.96 ± 12.11	< 0.001	77.19 ± 12.13	< 0.001	76.72 ± 12.26	< 0.001
FPG (mmol/L)	4.78 ± 1.13	5.01 ± 1.41	< 0.001	4.99 ± 1.39	0.001	4.93 ± 1.36	0.032
TG (mmol/L)	1.79 ± 1.71	2.01 ± 1.81	< 0.001	1.98 ± 2.04	0.032	2.05 ± 1.23	0.013
TC (mmol/L)	5.35 ± 1.12	5.44 ± 1.12	0.020	5.44 ± 1.21	0.093	5.53 ± 1.10	0.009
HDL-C (mmol/L)	1.32 ± 0.33	1.26 ± 0.30	< 0.001	1.28 ± 0.31	0.003	1.24 ± 0.28	< 0.001
LDL-C (mmol/L)	3.19 ± 0.86	3.27 ± 0.86	0.011	3.25 ± 0.84	0.154	3.36 ± 0.92	0.002
ALT (IU/L)	29.25 ± 29.65	32.23 ± 27.51	0.004	31.86 ± 22.61	0.065	32.85 ± 25.87	0.051
AST (IU/L)	27.24 ± 16.19	28.89 ± 17.63	0.007	28.75 ± 11.80	0.049	28.57 ± 13.43	0.184
NAFLD	488 (17.9)	291 (24.6)	< 0.001	122 (27.4)	0.002	79 (22.6)	0.001
MS	246 (10.1)	180 (15.6)	< 0.001	73 (15.5)	0.005	44 (16.4)	0.016
Smoking	662 (26.6)	373 (31.7)	0.002	151 (30.5)	0.087	101 (35.2)	0.004
Alcohol	854 (34.4)	445 (37.8)	0.042	191 (38.6)	0.078	118 (41.1)	0.028

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; TG: Triglyceride; TC: Total cholesterol; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease; MS: Metabolic syndrome.

to the four knowledgeable ultrasound criteria (liver brightness, deep attenuation, hepatorenal echo contrast and vascular blurring), subjects who met hepatorenal contrast and liver brightness were diagnosed with fatty liver disease[26]. NAFLD was diagnosed based on the guidelines for assessment and management of NAFLD in the Asia-Pacific region when fatty liver was present in the absence of the following: (1) viral hepatitis (hepatitis B or hepatitis C); (2) hepatic cirrhosis and liver carcinoma; (3) autoimmune liver disease or other liver disease; and (4) excess alcohol consumption (> 140 g/wk for men or > 70 g/wk for women)[27].

The definition of metabolic syndrome (MS) was based on the presence of three or more of the following China Diabetes Federation MS criteria: (1) central obesity: BMI ⩾ 25 in both genders; (2) hypertension: SBP ⩾ 140 mmHg or DBP ⩾ 90 mmHg or previously diagnosed; (3) dyslipidemia: TG ⩾ 1.7 mmol/L and/or HDL-C < 0.9 mmol/L in males and < 1.0 mmol/L in females; and (4) hyperglycemia: FPG ⩾ 6.1 mmol/L or hyperglycemia previously diagnosed[28].

Statistical analysis

Statistical analyses were conducted using SPSS software (SPSS version 23.0 for Windows). Demographic data and other risk factors for colorectal polyps, adenomatous polyps and hyperplastic polyps are presented as the mean ± SD or percentages. One-way analysis of variance (ANOVA) or Kruskal-Wallis test for continuous variables and Pearson χ^2 tests for categorical variables were used to compare the baseline of the study population according to the groups that had been classified as being polyp-free or having colorectal polyps, adenomatous polyps, and hyperplastic polyps. The associations between NAFLD and the prevalence of colorectal polyps, adenomatous polyps and hyperplastic polyps were analyzed by multinomial logistic regression after adjustment for independent variables, including NAFLD, age, smoking, alcohol and MS. In addition, polytomous logistic models were used for associations between NAFLD and multiple outcomes, such as number of polyps (single or multiple), size of polyps (small: < 1 cm or large: ⩾ 1 cm) and location of polyps (proximal lesions, distal lesions and bilateral lesions), in the groups of adenomatous polyps and hyperplastic polyps. All models were adjusted for age, smoking, alcohol and MS as confounding variables. A two-side *P* value less than 0.05 was considered statistically significant, and each OR is presented together with its 95%CI.

RESULTS

Subject characteristics

The demographic and clinical characteristics of eligible subjects according to colonoscopic findings are presented in Table 1. Among 3686 subjects in our study, 1185 (32.1%) were diagnosed with colorectal polyps, including 498 (13.5%) with adenomatous polyps, 288 (7.8%) with hyperplastic polyps, and 399 (10.8%) with inflammatory polyps. Of the 2430 male subjects, 1487 (61.2%) were in the normal group, and 943 (38.8%) were in the colorectal polyps group, including 393 (16.2%) with adenomatous polyps and 239 (9.8%) with hyperplastic polyps (Table 2). Of the female

Table 2 Demographic and clinical characteristics of 2430 males and 1256 females according to colonoscopic findings *n* (%)

Characteristic of males	Polyp-free	Colorectal polyps	*P* value *vs* Polyp-free	Adenomatous polyps	*P* value *vs* Polyp-free	Hyperplastic polyps	*P* value *vs* Polyp-free
Colonoscopic findings of 2430 males							
	1487 (61.2)	943 (38.8)		393 (16.2)		239 (9.8)	
Age (yr)	44.61 ± 9.91	50.18 ± 9.67	< 0.001	51.71 ± 9.54	< 0.001	47.56 ± 8.90	< 0.001
Weight (kg)	69.46 ± 10.18	70.88 ± 10.20	0.001	70.27 ± 10.12	0.164	71.47 ± 10.74	0.005
Height (cm)	169.13 ± 5.56	168.88 ± 5.80	0.305	168.28 ± 6.04	0.012	169.44 ± 5.72	0.427
BMI (kg/m^2)	24.28 ± 3.22	24.81 ± 3.06	< 0.001	24.77 ± 3.03	0.006	24.85 ± 3.22	0.011
SBP (mmHg)	127.29 ± 16.24	130.47 ± 17.83	< 0.001	131.97 ± 18.29	< 0.001	127.82 ± 16.88	0.639
DBP (mmHg)	76.44 ± 11.75	78.14 ± 12.03	0.001	78.77 ± 11.93	0.001	77.57 ± 12.11	0.173
FPG (mmol/L)	4.86 ± 1.26	5.06 ± 1.49	0.001	5.04 ± 1.46	0.012	4.96 ± 1.42	0.233
TG (mmol/L)	2.14 ± 2.04	2.15 ± 1.95	0.920	2.16 ± 2.24	0.878	2.14 ± 1.24	0.987
TC (mmol/L)	5.42 ± 1.16	5.42 ± 1.12	0.954	5.45 ± 1.24	0.686	5.49 ± 1.08	0.374
HDL-C (mmol/L)	1.22 ± 0.28	1.22 ± 0.29	0.892	1.24 ± 0.30	0.244	1.21 ± 0.27	0.597
LDL-C (mmol/L)	3.26 ± 0.86	3.26 ± 0.84	0.829	3.24 ± 0.83	0.632	3.34 ± 0.90	0.221
ALT (IU/L)	34.71 ± 33.63	35.31 ± 29.49	0.657	35.54 ± 23.59	0.649	35.2 ± 27.26	0.834
AST (IU/L)	29.00 ± 17.83	30.22 ± 19.06	0.115	30.37 ± 12.31	0.155	29.32 ± 13.41	0.796
NAFLD	355 (23.9)	268 (28.4)	0.014	116 (29.5)	0.028	72 (30.1)	0.038
MS	194 (13.3)	156 (17.0)	0.018	64 (15.8)	0.123	37 (18.3)	0.334
Smoking	465 (31.4)	327 (34.9)	0.076	127 (32.4)	0.717	91 (38.2)	0.045
Alcohol	577 (39.0)	377 (40.3)	0.536	153 (39.0)	0.995	100 (42.0)	0.379
Colonoscopic findings of 1256 females							
	1014 (80.7)	242 (19.3)		105 (8.4)		49 (3.9)	
Age (yr)	46.73 ± 9.97	51.68 ± 10.96	< 0.001	51.99 ± 10.68	< 0.001	51.10 ± 9.50	0.003
Weight (kg)	56.62 ± 8.03	58.41 ± 8.27	0.002	57.34 ± 8.61	0.385	60.42 ± 7.01	0.001
Height (cm)	158.04 ± 5.32	157.62 ± 5.16	0.268	157.08 ± 5.46	0.080	157.89 ± 4.21	0.840
BMI (kg/m^2)	22.66 ± 2.96	23.50 ± 3.05	< 0.001	23.21 ± 3.06	0.073	24.24 ± 2.66	< 0.001
SBP (mmHg)	120.23 ± 19.07	128.13 ± 20.13	< 0.001	125.21 ± 19.59	0.012	127.25 ± 22.56	0.013
DBP (mmHg)	69.12 ± 11.70	72.40 ± 11.31	< 0.001	71.19 ± 11.03	0.086	72.63 ± 12.27	0.041
FPG (mmol/L)	4.66 ± 0.90	4.80 ± 0.99	0.037	4.80 ± 1.07	0.147	4.80 ± 1.06	0.318
TG (mmol/L)	1.27 ± 0.80	1.46 ± 0.92	0.002	1.30 ± 0.66	0.716	1.65 ± 1.11	0.002
TC (mmol/L)	5.25 ± 1.05	5.52 ± 1.10	< 0.001	5.43 ± 1.09	0.088	5.72 ± 1.17	0.002
HDL-C (mmol/L)	1.48 ± 0.33	1.42 ± 0.31	0.008	1.43 ± 0.31	0.114	1.41 ± 0.28	0.123
LDL-C (mmol/L)	3.09 ± 0.85	3.33 ± 0.91	< 0.001	3.30 ± 0.87	0.017	3.45 ± 1.00	0.004
ALT (IU/L)	21.15 ± 19.93	20.13 ± 11.46	0.451	17.74 ± 9.29	3.000	21.76 ± 13.28	0.835
AST (IU/L)	24.64 ± 12.98	23.71 ± 8.56	0.286	22.62 ± 6.73	0.121	25.00 ± 13.10	0.848
NAFLD	93 (9.2)	23 (9.5)	0.873	6 (5.7)	0.160	7 (14.3)	0.324
MS	52 (5.3)	24 (10.2)	0.021	9 (8.8)	0.227	7 (14.3)	0.084
Smoking	197 (19.6)	46 (19.2)	0.884	24 (23.3)	0.369	10 (20.4)	0.887
Alcohol	277 (27.5)	68 (28.3)	0.804	38 (36.9)	0.063	18 (36.7)	0.201

BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FPG: Fasting plasma glucose; TG: Triglyceride; TC: Total cholesterol; HDL-C: High density lipoprotein-cholesterol; LDL-C: Low density lipoprotein-cholesterol; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NAFLD: Non-alcoholic fatty liver disease; MS: Metabolic syndrome.

subjects, 1014 (80.7%) were in the normal group, and 242 (19.3%) were in the colorectal polyps group, including 105 (8.4%) with adenomatous polyps and 49 (3.9%) with hyperplastic polyps. The prevalence of NAFLD in the colorectal polyps group, adenomatous polyps group, and hyperplastic polyps group were 268 (28.4%), 116 (29.5%), and 72 (30.1%) in males and 23 (9.5%), 6 (5.7%), and 7 (14.3%) in females, respectively. Overall, subjects without colorectal polyps had younger age, higher HDL-C, and reduced weight, BMI values, SBP, DBP, FPG, TG, TC, LDL-C, MS prevalence, smoking and alcohol use.

NAFLD is related to the prevalence of adenomatous polyps and hyperplastic polyps

The results of the multinomial logistic regression of risk factors for colorectal polyps, adenomatous polyps and hyperplastic polyps are presented in Table 3. In model A, compared with the polyp-free group, multinomial logistic regression demonstrated that subjects with NAFLD exhibited an increased prevalence of colorectal polyps (OR = 1.49, 95%CI: 1.26-1.76, $P < 0.001$), adenomatous polyps (OR = 1.49, 95%CI: 1.18-1.87, $P < 0.05$) and hyperplastic polyps (OR = 1.73, 95%CI: 1.31-2.29, $P < 0.001$) compared with subjects without NAFLD. In model C, the adjusted ORs for colorectal polyps (OR = 1.26, 95%CI: 1.05-1.51, $P < 0.05$), adenomatous polyps (OR = 1.28, 95%CI: 1.01-1.64, $P < 0.05$) and hyperplastic polyps (OR = 1.35, 95%CI: 1.01-1.82, $P < 0.05$) remained significantly increased in subjects with NAFLD.

Does the association between NAFLD and colorectal polyps persist after sex analysis?

Multinomial logistic regression was performed to obtain a deeper understanding of the relationship between

Table 3 Adjusted ORs and 95%CIs of clinical variables on the risk of colorectal polyps, adenomatous polyps and hyperplastic polyps based on multinomial logistic regression

Polyps	Model A OR (95%CI)	Model B OR (95%CI)	Model C OR (95%CI)
Colorectal polyps			
NAFLD	1.49 (1.26-1.76)[b]	1.28 (1.07-1.52)[a]	1.26 (1.05-1.51)[a]
Gender		2.81 (2.37-3.34)[b]	2.76 (2.32-3.29)[b]
Age		1.06 (1.05-1.06)[b]	1.06 (1.05-1.06)[b]
Smoking		1.15 (0.98-1.35)	1.15 (0.97-1.38)
Alcohol			1.01 (0.86-1.20)
MS			1.12 (0.90-1.40)
Adenomatous polyps			
NAFLD	1.49 (1.18-1.87)[a]	1.29 (1.02-1.64)[a]	1.28 (1.01-1.64)[a]
Gender		2.83 (2.22-3.61)[b]	2.78 (2.18-3.55)[b]
Age		1.07 (1.06-1.08)[b]	1.07 (1.06-1.08)[b]
Smoking		1.09 (0.88-1.36)	1.06 (0.84-1.35)
Alcohol			1.09 (0.87-1.37)
MS			1.02 (0.76-1.38)
Hyperplastic polyps			
NAFLD	1.73 (1.31-2.29)[b]	1.40 (1.05-1.86)[a]	1.35 (1.01-1.82)[a]
Gender		3.23 (2.33-4.47)[b]	3.12 (2.25-4.33)[b]
Age		1.03 (1.02-1.05)[b]	1.03 (1.02-1.05)[b]
Smoking		1.30 (1.00-1.70)[a]	1.27 (0.95-1.70)
Alcohol			1.09 (0.82-1.44)
MS			1.13 (0.79-1.63)

Model A is univariate analysis; Model B is adjusted for gender, age and smoking; Model C is adjusted for gender, age, smoking, alcohol and MS. [a]$P < 0.05$; [b]$P < 0.001$. NAFLD: Non-alcoholic fatty liver disease; MS: Metabolic syndrome.

NAFLD and the prevalence of adenomatous polyps and hyperplastic polyps in males and females (Table 4). In model A, NAFLD was independently related to both adenomatous polyps (OR = 1.34, 95%CI: 1.04-1.71, $P < 0.05$) and hyperplastic polyps (OR = 1.38, 95%CI: 1.02-1.86, $P < 0.05$) in males, whereas no significant relationship was noted between NAFLD and colorectal polyps in females. After adjustment for age and smoking (model B), associations between NAFLD and adenomatous polyps (OR = 1.53, 95%CI: 1.19-1.98, $P < 0.05$) and hyperplastic polyps (OR = 1.44, 95%CI: 1.06-1.95, $P < 0.05$) were noted in males. In model C, after controlling for confounding factors, such as age, smoking, alcohol and MS, the associations between NAFLD and adenomatous polyps and hyperplastic polyps remained (OR = 1.53, 95%CI: 1.18-2.00, $P < 0.05$; OR = 1.42, 95%CI: 1.04-1.95, $P < 0.05$) statistically significant in males.

Stratified analysis

The stratified analysis performed according to the number, size and location of adenomatous polyps and hyperplastic polyps is presented in Table 5. In the adenomatous polyps group, compared with non-NAFLD and adjusted for age, smoking, alcohol and MS, NAFLD was associated with multiple adenomas (OR = 1.82, 95%CI: 1.29-2.55, $P = 0.001$), distal adenoma (OR = 1.63, 95%CI: 1.11-2.39, $P = 0.013$), and bilateral adenoma (OR = 1.89, 95%CI: 1.23-2.91, $P = 0.004$). The OR for large adenoma (OR = 2.13, 95%CI: 1.21-3.76, $P = 0.009$) increased by approximately 1.5-fold compared with small adenoma (OR = 1.44, 95%CI: 1.08-1.91, $P = 0.013$). In addition, the associations between NAFLD and the prevalence of single (OR = 1.81, 95%CI: 1.19-2.74, $P = 0.005$), small (OR = 1.45, 95%CI: 1.05-2.00, $P = 0.023$) and proximal (OR = 2.62, 95%CI: 1.32-5.17, $P = 0.006$) hyperplastic polyps were noticeably stronger.

DISCUSSION

Our study presented that individuals with NAFLD exhibited an increased prevalence of colorectal adenomatous polyps compared with individuals without NAFLD, which is consistent with previous reports[19,21]. This study also showed that NAFLD is a risk factor for the increased prevalence of hyperplastic polyps. Therefore, this is the first study performed to explore an association between ultrasound-diagnosed NAFLD and colorectal adenomatous and hyperplastic polyps.

In our study, for the entire enrolled population, NAFLD is a risk factor for the prevalence of adenomatous polyps (OR = 1.28) and hyperplastic polyps (OR = 1.35). To analyze whether sex influences the association of NAFLD with adenomatous and hyperplastic polyps, we presented data classified according to sex and demonstrated that NAFLD is a risk factor for adenomatous (OR = 1.53) and hyperplastic polyps (OR = 1.42) in males after considering confounding variables, such as age, smoking, alcohol and MS. In contrast, no association was noted between NAFLD and adenomatous and hyperplastic polyps in females. In the adenomatous polyps group, individuals with NAFLD had an increased risk of multiple, including small and large, distal and bilateral polyps. Furthermore, the association between NAFLD and hyperplastic polyps was more prominent for the risk of single, small and proximal hyperplastic polyps. Our results may explain why Touzin *et al*[20] failed to demonstrate that patients with biopsy-proven NAFLD had an increased incidence of colorectal adenoma, with only 233 (48% in females) patients enrolled.

The significant association between NAFLD and colorectal adenomatous polyps has been recognized by several researchers. Hwang *et al*[19] conducted a cross-sectional study and demonstrated that NAFLD is an independent risk factor for the prevalence of colorectal adenoma and particularly associated with an increased risk for multiple adenomas. Furthermore, Stadlmayr *et al*[21] examined 1200 patients *via* screening colonoscopy and identified an increased prevalence of colorectal adenomas and early colorectal cancer in patients with NAFLD compared with those without NAFLD. This study also reported that the prevalence rate of colorectal adenomas was obviously increased in males with NAFLD compared with females with NAFLD. In addition, Wong *et al*[29] conducted a cross-sectional study of 433 subjects, including 135 with biopsy-proven NAFLD, and demonstrated that patients with

Table 4 Adjusted odds ratios and 95%CIs of clinical variables on the risk of colorectal polyps, adenomatous polyps and hyperplastic polyps according to different-sex groups based on multinomial logistic regression

Polyps	Model A		Model B		Model C	
	Male	Female	Male	Female	Male	Female
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
Colorectal polyps						
NAFLD	1.27 (1.05-1.52)[a]	1.04 (0.64-1.68)	1.41 (1.16-1.71)[a]	0.80 (0.49-1.32)	1.40 (1.14-1.71)[a]	0.78 (0.47-1.28)
Age			1.06 (1.05-1.07)[b]	1.05 (1.03-1.06)[b]	1.06 (1.05-1.07)[b]	1.05 (1.03-1.06)[b]
Smoking			1.22 (1.02-1.46)[a]	0.95 (0.66-1.36)	1.23 (1.02-1.50)[a]	0.88 (0.57-1.36)
Alcohol					0.98 (0.81-1.19)	1.19 (0.81-1.74)
MS					1.07 (0.84-1.37)	1.37 (0.80-2.34)
Adenomatous polyps						
NAFLD	1.34 (1.04-1.71)[a]	0.60 (0.26-1.41)	1.53 (1.19-1.98)[a]	0.45 (0.19-1.07)	1.53 (1.18-2.00)[a]	0.44 (0.18-1.04)
Age			1.08 (1.06-1.09)[b]	1.05 (1.03-1.07)[b]	1.08 (1.06-1.09)[b]	1.05 (1.03-1.07)[b]
Smoking			1.10 (0.86-1.40)	1.24 (0.76-2.02)	1.12 (0.85-1.46)	0.90 (0.50-1.61)
Alcohol					0.97 (0.75-1.25)	1.77 (1.06-2.96)[a]
MS					0.98 (0.71-1.36)	1.17 (0.53-2.56)
Hyperplastic polyps						
NAFLD	1.38 (1.02-1.86)[a]	1.65 (0.72-3.78)	1.44 (1.06-1.95)[a]	1.30 (0.56-3.03)	1.42 (1.04-1.95)[a]	1.18 (0.50-2.78)
Age			1.04 (1.02-1.05)[b]	1.04 (1.01-1.07)[a]	1.04 (1.02-1.05)[b]	1.04 (1.01-1.07)[a]
Smoking			1.38 (1.03-1.83)[a]	1.01 (0.49-2.06)	1.38 (1.01-1.88)[a]	0.71 (0.31-1.65)
Alcohol					1.01 (0.74-1.36)	1.85 (0.92-3.72)
MS					1.02 (0.69-1.52)	2.01 (0.81-4.94)

Model A is univariate analysis; Model B is adjusted for age and smoking; Model C is adjusted for age, smoking, alcohol and MS. [a]$P < 0.05$, [b]$P < 0.001$. NAFLD: Non-alcoholic fatty liver disease; MS: Metabolic syndrome.

Table 5 Adjusted odds ratios and 95%CIs of non-alcoholic fatty liver disease for adenomatous polyps and hyperplastic polyps of the colon and rectum by morphological characteristics in males

	Adenomatous polyps		*P* value	Hyperplastic polyps		*P* value
	Cases	OR (95%CI)		Cases	OR (95%CI)	
Number						
Single	186	1.27 (0.88-1.82)	0.205	117	1.81 (1.19-2.74)	0.005
Multiple	207	1.82 (1.29-2.55)	0.001	121	1.13 (0.73-1.76)	0.583
Size						
Small (< 1 cm)	332	1.44 (1.08-1.91)	0.013	229	1.45 (1.05-2.00)	0.023
Large (≥ 1 cm)	61	2.13 (1.21-3.76)	0.009	9	1.00 (0.20-5.07)	0.999
Location						
Proximal lesions	129	1.15 (0.73-1.80)	0.539	38	2.62 (1.32-5.17)	0.006
Distal lesions	146	1.63 (1.11-2.39)	0.013	156	1.33 (0.91-1.95)	0.146
Bilateral lesions	118	1.89 (1.23-2.91)	0.004	44	1.05 (0.51-2.17)	0.894

ORs compared with non-alcoholic fatty liver disease and adjusted for age, smoking, alcohol and metabolic syndrome.

NAFLD exhibited an increased prevalence of adenomas (34.7% *vs* 21.5%, $P = 0.043$). Among biopsy-proven NAFLD patients, patients with non-alcoholic steatohepatitis exhibited an increased prevalence of adenomas (51.0% *vs* 25.6%, $P = 0.005$).

Currently, the mechanisms responsible for the relationship between NAFLD and colorectal adenomas have not been clearly understood. The potential mechanisms for how NAFLD promotes the development of colorectal adenomas include insulin resistance and chronic inflammation[30,31]. Insulin and IGF may facilitate the development of colorectal adenoma through their antiapoptotic and proliferative effects[31]. It is generally accepted that adipocytokine metabolism disorders, which influence the development of colorectal neoplasms, exist in NAFLD individuals[32,33]. In addition, as an adipocyte-secreted factor, monocyte chemotactic protein-1 attracts macrophages and causes local inflammatory response and the release of other cytokines[34]. Furthermore, inflammatory cytokines, such as interleukin-6, tumor necrosis factor-alpha and adiponectin, play an important role in the development of colorectal adenomas in NAFLD patients. The levels of interleukin-6 and tumor necrosis factor-alpha, as carcinogens and growth-promoting factors, are increasing. In contrast, the levels of adiponectin, a protective factor, are reduced with NAFLD[35].

Although hyperplastic polyps have been regarded as non-neoplastic polyps lacking malignant potential, recent studies provided some evidence of the presence of molecular changes consistent with neoplasia, including ras mutations, hypomethylation of the c-myc gene and high levels of microsatellite instability[36-41]. Furthermore, Huang *et al*[11] conducted a retrospective cohort study using the records of a single surgeon's colonoscopic experience over a 20-year period and

found that patients with hyperplastic polyps on initial colonoscopic examination may have twice the risk of adenomas on follow-up colonoscopy compared with those with clean initial examination. Hyman *et al*[42] found that 7 (54%) of 13 patients with hyperplastic polyposis developed colorectal cancer during their study period and suggested that patients with hyperplastic polyposis are at high risk for colorectal cancer. A cross-sectional study suggested that higher serum insulin levels were associated with an increased risk of proximal colon adenomatous and hyperplastic polyps[8]. An experimental study reported that hyperinsulinemia was associated with high susceptibility to colonic carcinogenesis induced by DNA methylation in animals[43]. Furthermore, the roles of insulin resistance and hyperinsulinemia in NAFLD are well recognized[44,45]. Given the link between NAFLD and hyperinsulinemia and the link between hyperinsulinemia and hyperplastic polyps, our study clearly demonstrated that NAFLD was associated with hyperplastic polyps.

Some studies demonstrated that the association of dyslipidemia and colon adenoma existed in different sex groups. Kim *et al*[23] supported that the risk of adenoma increased significantly by 2-fold when TC $\geqslant$ 150 mg/dL or HDL-C < 40 mg/dL in males. In contrast, no association was noted between adenoma prevalence and dyslipidemia in females. In addition, Liu *et al*[22] declared that dyslipidemia and large waist, as a component of MS, exhibited an association with colon adenoma in males but not in females. As a metabolic disease, NAFLD is closely correlated with dyslipidemia. The relationship between NAFLD and colorectal polyps may not be consistent in different sex groups.

Our study had several limitations that should be acknowledged. First, potential selection bias might exist based on general health check-ups, and data, such as visceral fat, fat intake and intestinal flora, are insufficient. Second, the causality between NAFLD and the risk for colorectal adenomatous and hyperplastic polyps is difficult to infer through a cross-sectional study. Third, our study subjects were derived from a single center, and multi-centered research should be performed to further confirm the association in the next step. Fourth, although liver biopsy is a standard criterion for NAFLD diagnosis, the diagnosis of fatty liver was based on ultrasound imaging with a 94% sensitivity and 84% specificity[46].

In conclusion, our study clearly demonstrated that NAFLD is specifically associated with an increased risk of colorectal adenomatous and hyperplastic polyps in men. However, NAFLD is not a significant factor in the prevalence of colorectal polyps in women.

COMMENTS

Background

Colorectal adenomatous polyps and some subsets of hyperplastic polyps have been considered precursors of hereditary and sporadic colorectal cancer. Previous studies demonstrated that non-alcoholic fatty liver disease (NAFLD) is associated with an increased risk of colorectal adenoma. However, the association of NAFLD and hyperplastic polyps remains unclear.

Research frontiers

Colorectal cancer arises from colorectal adenomas and some subsets of hyperplastic polyps. Early diagnosis of colorectal adenomatous and hyperplastic polyps is very important to reduce mortality. It is necessary for males with NAFLD to undergo colonoscopy screening and surveillance.

Innovations and breakthroughs

This study identified NAFLD as an independent risk factor for colorectal adenomatous and hyperplastic polyps in males, and NAFLD may be not a significant factor in the prevalence of colorectal polyps in women.

Applications

This research demonstrated that males who have NAFLD exhibit an increased risk of colorectal adenomatous and hyperplastic polyps. The prevalence of colorectal adenocarcinoma is high in men. Therefore, it is necessary for males with NAFLD to undergo colonoscopy screening and surveillance.

Peer-review

The current manuscript investigated features of the metabolic syndrome (presence of NAFLD) and incidence of adenomatous and hyperplastic polyps.

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ORIGINAL ARTICLE

Observational Study

Development and validation of a simple and multifaceted instrument, GERD-TEST, for the clinical evaluation of gastroesophageal reflux and dyspeptic symptoms

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Abstract

AIM

To evaluate the psychometric properties of a newly developed questionnaire, known as the gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test (GERD-TEST), in patients with GERD.

METHODS

Japanese patients with predominant GERD symptoms recruited according to the Montreal definition were treated for 4 wk using a standard dose of proton pump inhibitor (PPI). The GERD-TEST and the Medical Outcome Study Short Form-8 Health Survey (SF-8) were administered at baseline and after 4 wk of treatment. The GERD-TEST contains three domains: the severity of GERD and functional dyspepsia (FD) symptoms (5 items), the level of dissatisfaction with daily life (DS) (4 items), and the therapeutic efficacy as assessed by the patients and medication compliance (4 items).

RESULTS

A total of 290 patients were eligible at baseline; 198 of these patients completed 4 wk of PPI therapy. The internal consistency reliability as evaluated using the Cronbach's α values for the GERD, FD and DS subscales ranged from 0.75 to 0.82. The scores for the GERD, FD and DS items/subscales were significantly correlated with the physical and mental component summary scores of the SF-8. After 4 wk of PPI treatment, the scores for the GERD items/subscales were greatly reduced, ranging in value from 1.51 to 1.87 and with a large effect size ($P < 0.0001$, Cohen's d; 1.29-1.63). Statistically significant differences in the changes in the scores for the GERD items/subscales were observed between treatment responders and non-responders ($P < 0.0001$).

CONCLUSION

The GERD-TEST has a good reliability, a good convergent and concurrent validity, and is responsive to the effects of treatment. The GERD-TEST is a simple, easy to understand, and multifaceted PRO instrument applicable to both clinical trials and the primary care of GERD patients.

Key words: Gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test; Patient-reported outcome; Gastroesophageal reflux disease; Validity; Reliability

Core tip: A patient-reported outcome (PRO) can be a clinically relevant outcome measure of disease impact and treatment response in both clinical trials and primary care. The practical use and dissemination of PRO as a diagnostic and evaluation tool is anticipated; however, most PROs are lengthy and complicated. Therefore, we developed a simple, easy-to-understand and multifaceted PRO instrument, the gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test (GERD-TEST). The psychometric characteristics of the GERD-TEST were excellent, demonstrating good validity and reliability. The GERD-TEST enabled a multifaceted evaluation not only of the severity of symptoms, but also of the impact of the symptoms on daily life, the therapeutic response as assessed by the patient. The GERD-TEST is expected to be a useful diagnostic/treatment tool for both clinical research and in daily clinical practice settings.

Nakada K, Matsuhashi N, Iwakiri K, Oshio A, Joh T, Higuchi K, Haruma K. Development and validation of a simple and multifaceted instrument, GERD-TEST, for the clinical evaluation of gastroesophageal reflux and dyspeptic symptoms. *World J Gastroenterol* 2017; 23(28): 5216-5228 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5216.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5216

INTRODUCTION

Gastroesophageal reflux disease (GERD) is defined as a condition that develops when the reflux of stomach contents causes troublesome symptoms and/or complications, according to the Montreal definition[1]. GERD is a chronic condition that interferes with various aspects of daily life such as eating, sleeping, daily activities and mood. GERD is one of the most common disorders treated in primary care, and its overall prevalence appears to have increased in Japan recent years[2-4].

GERD, even without any complications, poses a problem in that the symptoms of the disease interfere with various aspects of daily living, thereby lowering the quality of life (QOL) of the patient[5,6]. It is important, therefore, to diagnose patients appropriately and to treat patients efficiently.

Reportedly, concurrent functional dyspepsia (FD) is frequently encountered in patients with GERD[7-12]. FD is also generally recognized as having an untoward effect on a patient's daily living, with a consequent reduction in QOL[13-15]. Thus, the possible presence of concurrent manifestations of FD should be considered even in patients seeking medical advice for GERD symptoms, and if FD symptoms are present, they should be treated appropriately and at the same time.

The importance of patient-reported outcome (PRO) in evaluating medical care has been stressed in recent years[16-20]. The Food and Drug Administration (FDA) guidance[16] recommends the use of an appropriate PRO measure with proven reliability and validity for the treatment of disorders in which the treatment goal is to ameliorate symptoms. The application of PRO not only in clinical trials, but also in daily clinical practice

settings would enable greater objectivity in the diagnosis and evaluation of therapeutic responses in GERD cases and the provision of effective and efficient treatment. However, an optimal PRO for GERD patients does not presently exist. Most of the previously developed PROs for GERD were too long or were too complicated to use in routine clinical care, and most were not well validated for the diagnosis of GERD, the evaluation of symptom-induced burden, the impact on daily life, or the therapeutic response. The lack of a simple, easy to understand instrument for GERD patients encouraged the development of the presently reported gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test (GERD-TEST).

The concepts behind the newly developed questionnaire, known as the GERD-TEST, were as follows: (1) Simplicity (*i.e.*, a minimum number of items), (2) easy to understand; (3) applicability to the diagnosis of GERD and the evaluation of symptom-induced burden, impact on daily life, and therapeutic response after treatment; (4) the ability to detect simultaneous FD; and (5) applicability to both clinical trials and primary care.

The aim of the present study was to assess the reliability and validity of the GERD-TEST in a population of patients who had been diagnosed as having GERD according to the Montreal definition.

MATERIALS AND METHODS

Study design

This was a multicenter, prospective, observational study conducted at 29 institutions in Japan, in which one or more investigators per institution was a member of the GERD Society, a Japanese collaborative research group consisting of experts in clinical practice of GERD. The study was conducted in accordance with the Declaration of Helsinki (sixth revision, 2008), after approval by the ethics committee of each institution or the central ethics committee of Nishi Clinic, Osaka, Japan. The study was registered with the University Hospital Medical Information Network Center Clinical Trials Registry in Japan (reference number UMIN000006614).

Patients

Outpatients with symptomatic GERD who received proton pump inhibitor (PPI) treatment in routine clinical care were recruited for this study. After endoscopic examination, patients were treated with a PPI at a dosage approved in Japan before the start of this study (April 2011), *i.e.*, omeprazole 20 mg once daily, lansoprazole 30 mg once daily, or rabeprazole 10 or 20 mg once daily.

Inclusion criteria were as follows: (1) moderate or severe heartburn or acid regurgitation at least once a week or mild heartburn or acid regurgitation at least twice a week during the 2 wk prior to the start of the study (the Montreal definition); (2) at least 20 years of age; and (3) provision of written informed consent.

Exclusion criteria were (1) comorbidity or history of disease that could potentially affect the study results [for example, Zollinger-Ellison syndrome, inflammatory bowel disease, irritable bowel syndrome (IBS), esophageal stricture, eosinophilic esophagitis, achalasia, malabsorption, or cerebrovascular disease]; (2) concurrent symptoms of concern such as vomiting, peptic ulcer except those in the scarred stage, and severe hepatic or renal or cardiac diseases, mental disorder, uncontrolled metabolic diseases, neurological diseases, collagen diseases, or other diseases; (3) confirmed or suspected malignancy; (4) history of gastrointestinal tract resection or vagotomy; (5) history of hypersensitivity to PPIs or their excipients; (6) *Helicobacter pylori* eradication within 6 mo before enrollment; (7) pregnancy, possible pregnancy, or breastfeeding; (8) ingestion of PPI or histamine type 2 (H2)-receptor antagonist within 1 wk of enrollment; and (9) patients otherwise deemed to be ineligible by the attending physician.

Prohibited concomitant drugs were those that might affect the study results (PPIs other than the study drugs, H2-receptor antagonists, prokinetic agents, gastric mucosal protective agents, and anticholinergic drugs), and drugs that might interact with the study drugs.

Assessments

Severity of reflux esophagitis was assessed according to the modified Los Angeles classification system[21,22]. Patients' demographic and clinical characteristics were recorded before beginning PPI therapy (0w) with a series of questionnaires. GERD and dyspeptic symptoms and QOL were assessed using the GERD-TEST[23] and the acute (1-wk-recall) version of a health-related QOL survey (SF-8)[24], respectively, at 0 wk, 2 wk, and 4 wk after PPI treatment. Psychiatric bias was assessed using the Hospital Anxiety and Depression Scale[25] at 0 wk and 4 wk. All questionnaires were completed and mailed to the data center by the study participants.

Questionnaires for data collection

Patient characteristics were recorded using a questionnaire that included sex, age, height, weight, and lifestyle factors (regularity of daily life, consumption of caffeine-containing beverages or high-fat meals, smoking status, and alcohol consumption).

The GERD-TEST is a patient-reported questionnaire composed of 13 items for investigating GERD and dyspepsia symptoms, impact to the patient's daily life, and patient's impression of the therapy. Questions (Q) 1 to Q5 of the GERD-TEST assess the severity of upper abdominal symptoms; Q6-Q9 assess the impact of symptoms on daily life, including eating, sleeping, daily activity, and mood; Q10-Q12 evaluate the therapeutic response to the PPIs; Q13 asks compliance with the

Table 1 Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test

Q1. Have you been bothered by heartburn during the past week?
(By heartburn we mean a burning pain or discomfort behind the breastbone in your chest)
Q2. Have you been bothered by acid regurgitation during the past week?
(By acid regurgitation we mean regurgitation or flow of sour or bitter fluid into your mouth)
Q3. Have you been bothered by epigastric pain or burning during the past week?
(Epigastric pain includes any type of pain of the stomach)
Q4. Have you been bothered by postprandial fullness during the past week?
(Postprandial fullness refers to discomfort or a sensation of heaviness caused by the food you consume remaining in the stomach)
Q5. Have you been bothered by early satiation during the past week? (Early satiation refers to the inability to finish a normally sized meal)
Response scale for Q1-5:
1 = no discomfort at all, 2 = slight discomfort, 3 = mild discomfort, 4 = moderate discomfort, 5 = moderately severe discomfort, 6 = severe discomfort, 7 = very severe discomfort.
Q6. During the past week, how often have you felt dissatisfaction because you were unable to eat meals as you intended due to chest and stomach symptoms?
(Not being able to eat as you intended refers to the inability to eat the sufficient amount of food you want to eat at an uninhibited, natural pace)
Q7. During the past week, how often have you felt dissatisfaction due to impaired sleep caused by chest and stomach symptoms?
Q8. During the past week, how often have you felt dissatisfaction due to impairment of your work, housework, or other daily activities caused by chest and stomach symptoms?
Q9. During the past week, how often have you felt dissatisfaction because you were in a bad mood due to chest and stomach symptoms?
Response scale for Q.6-9:
1 = not at all, 2 = slightly, 3 = moderately, 4 = quite a lot, 5 = extremely.
Q10. During the past week, how often have you wanted another drug in addition to the drug your doctor prescribed because of intense symptoms of heartburn and acid regurgitation?
1 = not at all, 2 = on 1 d, 3 = on 2 to 3 d, 4 = on 4 to 5 d, 5 = always.
Q11. During the past week, how have you felt about symptoms of heartburn and acid regurgitation as compared with the symptom severity before current treatment?
1 = extremely improved, 2 = improved, 3 = slightly improved, 4 = not changed, 5 = aggravated.

Q12. If 10 corresponds to your symptoms before current treatment and 0 is "symptom-free", what number corresponds to symptoms of heartburn and acid regurgitation during the past week? Please circle the applicable score below:
0 …….. 1 …….. 2 ……..... 3 ……..... 4……..... 5 ……..... 6 ……..... 7 …..…….. 8 ……..... 9 ……..... 10
| |
Symptom-free — Symptoms before current treatment
Q13. What proportion of the proton pump inhibitor prescribed to you did you take as instructed?
1 = took drug as instructed, 2 = generally took drug as instructed (took at least three-quarters of the drug prescribed), 3 = sometimes forgot (took at least half but less than three-quarters of the drug prescribed, 4 = took little (took less than half of the drug prescribed), 5 = did not take any.

Before therapy, questions about treatment efficacy and adherence (Q10–Q13) were excluded. The following scores were defined: Score of GERD symptom subscale (GERD-SS) = (Q1 + Q2)/2; Score of Epigastric pain/burning symptom (EPS-Sx) = Q3; Score of Postprandial distress symptom subscale (PDS-SS) = (Q4 + Q5)/2; Score of FD symptom subscale (FD-SS) = [Q3 + (Q4 + Q5)/2]/2; Score of dissatisfaction with daily life subscale (DS-SS) = (Q6 + Q7 + Q8 + Q9)/4; Residual symptom rate (%) = 100 × (GERD-SS score at 4 wk-1)/(GERD-SS score at 0 wk-1). GERD: Gastroesophageal reflux and dyspepsia-therapeutic efficacy; FD: Functional dyspepsia.

medication; Q1-Q11 and Q13 use a Likert scale; Q12 uses an numeric rating scale (NRS) (Table 1).

The SF-8 is a generic questionnaire used to investigate health status and is composed of a physical component summary (PCS) and a mental component summary (MCS)[20]. These scores are normalized to the general population, with higher scores indicating better physical and mental QOL, with a normative score of 50 and a SD of 10.

Definitions of subscale scores in GERD-TEST

The GERD-SS was defined as the mean of scores for heartburn (Q1) and regurgitation (Q2). The FD-SS was defined as the mean of scores for epigastric pain/burning (Q3) and postprandial distress symptoms (the mean of scores for postprandial fullness [Q4] and early satiation [Q5]). The dissatisfaction with daily life (DS)-SS defined as the mean of scores for dissatisfaction with eating (Q6), sleeping (Q7), daily activities (Q8) and mood (Q9).

Outcome measures

To assess the therapeutic response to PPI in patients with GERD, three outcome measures were used, as follows: (1) Residual symptom rate of GERD-SS, which was calculated as 100 (%) × (GERD-SS score at 4 wk-1)/(GERD-SS score 0 wk-1), and therefore was 100% when GERD-SS score at 4 wk equaled that at 0 wk, and was 0% when the patient had no symptoms (a score of 1) at 4 wk. A higher residual symptom rate thus reflects a poorer response; (2) Patient's impression of therapy, which was the score for Q11 of GERD-TEST (*i.e.*, the score of impression of improvement in GERD symptoms as compared with the severity before taking current prescription, 1 for extremely improved, 2 for improved, 3 for slightly improved, 4 for not changed and 5 for aggravated); and (3) Relative GERD symptom intensity quantified

using an 11-point (*i.e.*, 0 for no symptoms to 10 for symptoms before taking current prescription).

Responder definition

The responder definition for each outcome measure was defined as follows, (1) residual symptom rate ≤ 50%; (2) patient's impression of improved or better; and (3) NRS ≤ 5, respectively.

Statistical analysis

Data analysis was undertaken using JMP10.0.2 software (SAS Institute Inc., Cary, NC, United States). All statistical tests were performed using a two-sided test with a significance level of 0.05.

Reliability

Cronbach's α is a coefficient of internal consistency that is commonly used as an estimate of the reliability of a psychometric test. Consequently, the Cronbach's α values were calculated from pairwise correlations between items to verify the internal consistency of the items in each subscale.

Convergent validity

Correlations between the scores for symptoms or dissatisfaction with daily life (DS) items/subscales and the PCS or MCS of the SF-8, as well as correlations between the scores for symptoms and DS items/subscales, were calculated in terms of the Pearson correlation coefficient (*r*), where values of $r \geqslant 0.100$, $\geqslant 0.300$, and $\geqslant 0.500$ were considered to be small, medium, and large effects, respectively[26].

Responsiveness and discriminate validity

The symptom and dissatisfaction scores obtained before and after therapy were compared using a paired *t*-test, and the symptom and DS scores at baseline and after 4 wk of PPI therapy and the changes in the scores before and after 4 wk of PPI therapy between responders and non-responders according to three different responder definitions were compared using unpaired *t*-tests. The effect sizes (Cohen's *d*) were then calculated, where Cohen's *d* values of $\geqslant 0.20$, $\geqslant 0.50$, and $\geqslant 0.80$ were considered to be small, medium, and large effects, respectively[26].

Specificity for differentiating between GERD and FD symptoms

To identify the types of symptoms that showed a response when therapeutic efficacy was assessed by the patients, multiple regression analyses were performed using the changes in scores for both the GERD-SS and the FD-SS before and after 4 wk of PPI therapy as explanatory variables; the outcome measures of the therapeutic response at 4 wk (*i.e.*, the patient's impression of the therapy [Q11] and the relative symptom intensity according to a NRS [Q12]) were used as objective variables. Interpretation of effect sizes were $\geqslant 0.1$ small, $\geqslant 0.3$ medium, and $\geqslant 0.5$ large in standardization coefficient of regression [β]; $\geqslant 0.02$ small, $\geqslant 0.13$ medium, and $\geqslant 0.26$ large in coefficient of determination [R^2].

RESULTS

Patient characteristics

A total of 290 patients were eligible at baseline; 178 (61%) were men, the mean age was 57.5 ± 13.9 years, and the mean body mass index (BMI) was 24.0 ± 3.9 kg/m^2. A diagnosis of erosive reflux disease (ERD) was made in 183 (63%) of the cases, while a diagnosis of nonerosive reflux disease (NERD) was made in 107 (37%) cases based on the results of an upper gastrointestinal endoscopy. Of these patients, 198 completed 4 wk of PPI therapy and were eligible for inclusion in the analysis; 126 (64%) of these patients were men, the mean age was 57.9 ± 13.1 years, and the mean BMI was 24.2 ± 4.1 kg/m^2. A diagnosis of ERD was made in 134 (68%) of the cases, and a diagnosis of NERD was made in 64 (32%) of the cases based on the results of an upper gastrointestinal endoscopy (Table 2).

Diagnostic accuracy of the GERD-TEST

Of the 290 symptomatic GERD patients who were recruited according to the Montreal definition, 246 (85%) were identified as GERD patients based on the results of the GERD-TEST (*i.e.*, the score for Q1 [heartburn] and/or Q2 [regurgitation] was $\geqslant 3$).

Reliability

The internal consistency of the items in each of the three subscales (GERD-SS, FD-SS and DS-SS) was acceptable, as shown by the Cronbach's α values (which ranged from 0.75 to 0.82) (Table 3).

Convergent validity

The Pearson's *r* for comparisons of the GERD-TEST with the SF-8 were used to assess convergent validity. There was a significant negative correlation between each of the GERD-TEST items/subscales and the PCS or MCS of the SF-8 [Pearson's *r* = (-0.19)-(-0.55)] (Table 4). In addition, a significant positive correlation was seen between each of the symptom items/subscales and the DS items/subscale of the GERD-TEST (Pearson's *r* = 0.32-0.72) (Table 4).

Therapeutic efficacy in GERD patients after 4 wk of PPI therapy

The GERD-TEST scores at baseline and after 4 wk of PPI therapy are shown in Figure 1. The distances between the lines on the graph show the score changes after treatment. The rates of responders after 4 wk of PPI therapy according to three different responder definitions were 79% for the "residual symptom rate ≤ 50%" definition (Figure 2), 79% for the "patient's

Table 2 Patients' characteristics *n* (%)

	At baseline (*n* = 290)	Accomplished 4W PPI Tx (*n* = 198)
Age (mean ± SD, yr)	57.5 ± 13.9	57.9 ± 13.1
Sex		
Male	178 (61)	126 (64)
Female	112 (39)	72 (36)
BMI (mean ± SD, kg/m^2)	24.0 ± 3.9	24.2 ± 4.1
Endoscopic findings		
NERD	107 (37)	64 (32)
Grade N	62 (21)	38 (19)
Grade M	45 (16)	26 (13)
ERD	183 (63)	134 (68)
Grade A	94 (32)	66 (33)
Grade B	60 (21)	47 (24)
Grade C	21 (7)	14 (7)
Grade D	8 (3)	7 (4)

NERD: Nonerosive reflux disease; ERD: Erosive reflux disease; PPI: Proton pump inhibitor.

Table 3 Internal consistency of each subscale for the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test (*n* = 290)

Subscales	Cronbach's α
GERD-SS	0.78
Heartburn	
Acid regurgitation	
FD-SS	0.75
Epigastric pain/burning	
Postprandial fullness	
Early satiation	
Dissatisfaction for daily life SS	0.82
Dissatisfaction for eating	
Dissatisfaction for sleeping	
Dissatisfaction for daily activity	
Dissatisfaction for the mood	

GERD: Gastroesophageal reflux and dyspepsia-therapeutic efficacy; FD: Functional dyspepsia.

impression of improved or better" definition (Figure 3), and 90% for the "NRS ⩽ 5" definition (Figure 4), respectively.

The GERD-TEST scores at baseline and after 4 wk of PPI therapy in responders and non-responders according to three different responder definitions are shown in Figures 5-7. The distance between the graph lines for baseline and after 4 wk of PPI therapy for both responders and non-responders show the score changes arising from treatment in the respective groups. The distances between the graph lines (*i.e.*, the score changes arising from treatment) were greater for responders than for non-responders as well as for GERD symptom items/subscales, compared with those for FD symptoms or DS (Figures 1, 5-7 and Tables 5-8).

Responsiveness

The responsiveness to PPI therapy was evaluated by comparing the scores for each GERD-TEST item/subscale between baseline and after 4 wk of PPI therapy. Significant differences were observed for all the GERD-TEST item/subscale scores between baseline and after 4 wk of PPI therapy, and the effect sizes, as determined using Cohen's *d*, were substantial (*i.e.*, 1.29-1.63 for GERD symptoms, 0.42-1.11 for FD symptoms, and 0.61-1.05 for dissatisfaction) (Table 5).

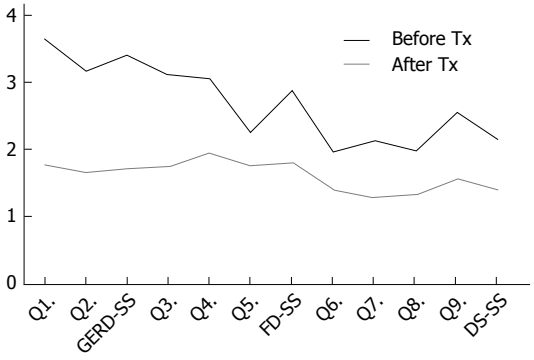

Figure 1 Changes in the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores at baseline and after 4 wk of proton pump inhibitor therapy. Q1: Heartburn; Q2: Regurgitation; Q3: Epigastric pain/burning; Q4: Postprandial fullness; Q5: Early satiation; Q6: Dissatisfaction with eating; Q7: Dissatisfaction with sleeping; Q8: Dissatisfaction with daily activity; Q9: Dissatisfaction with mood. DS-SS: Dissatisfaction with daily life subscale.

Concurrent validity

The concurrent validity of the GERD-TEST was evaluated by comparing the changes in the GERD-TEST scores of the treatment responders and those of the treatment non-responders according to three different responder definitions. The treatment responders demonstrated a statistically significant greater change in their scores than the treatment non-responders for all the GERD symptom items/subscale and for most of the FD and DS items/subscales (Tables 6-8).

Specificity for differentiating between GERD and FD symptoms

The results of a multiple regression analysis revealed that the GERD-SS score changes had larger β values than the FD-SS score changes for Q11 (0.371 *vs* 0.037) and Q12 (0.411 *vs* -0.092), reflecting the response to therapy and indicating that GERD symptoms can be well differentiated from FD symptoms in GERD patients.

DISCUSSION

Approximately 85% of reports from GERD patients recruited under the Montreal definition were diagnosed as having GERD based on the results of the GERD-TEST, providing evidence in support of the diagnostic usefulness of the GERD-TEST. The Cronbach's α for GERD-SS, FD-SS, and DS-SS in the GERD-TEST ranged from 0.75 to 0.82, indicating a superior internal consistency and high reliability. Significant correlations

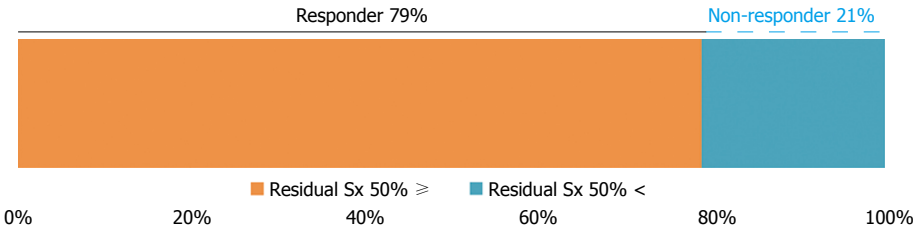

Figure 2 Rate of responders based on a "residual symptom rate ≤ 50%" definition after 4 wk of proton pump inhibitor therapy.

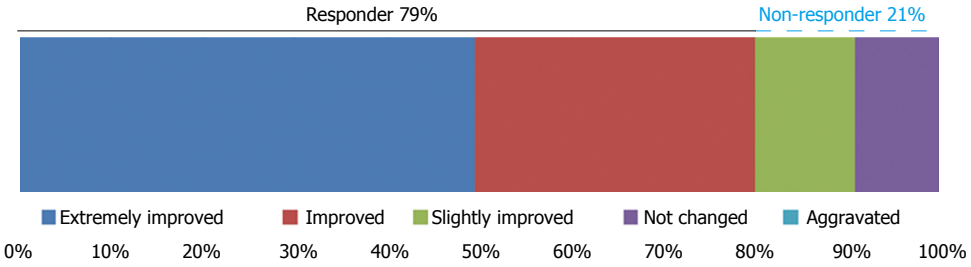

Figure 3 Distribution of patient's impressions of therapy (Q11) and the rate of responders based on a "patient's impression of improved or better" definition after 4 wk of proton pump inhibitor therapy.

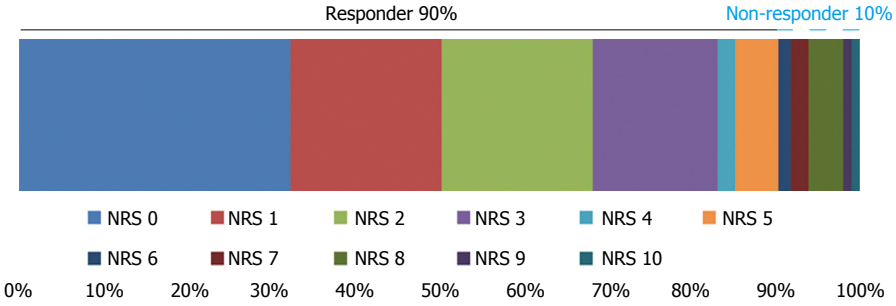

Figure 4 Distribution of numeric rating scale scores (Q12) and the rate of responders based on a "numeric rating scale ≤ 5" definition after 4 wk of proton pump inhibitor therapy.

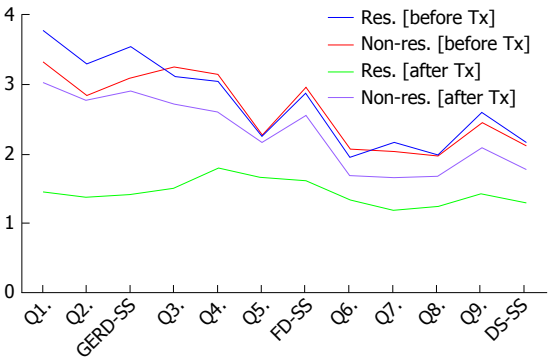

Figure 5 Changes in the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores of responders and non-responders based on a "residual symptom rate ≤ 50%" definition at baseline and after 4 wk of proton pump inhibitor therapy. Q1: Heartburn; Q2: Regurgitation; Q3: Epigastric pain/burning; Q4: Postprandial fullness; Q5: Early satiation; Q6: Dissatisfaction with eating; Q7: Dissatisfaction with sleeping; Q8: Dissatisfaction with daily activity; Q9: Dissatisfaction with mood. DS-SS: Dissatisfaction with daily life subscale.

were observed between symptom or living status items/subscales of the GERD-TEST and the PCS or MCS of the SF-8, demonstrating a good convergent validity. Both GERD and FD symptoms were seen to have a clear and consistently negative impact on the daily lives of patients, and this impact increased with increasing symptom severity (Table 4). There was a significant and marked reduction in GERD symptoms in response to the 4-wk PPI therapy. Improvements in FD symptoms and daily living status were also significant, though to a lesser extent than the amelioration of GERD symptoms. Thus, the responsiveness of the GERD-TEST to these improvements was gratifying. A comparison between responders and non-responders according to three definitions of responders (a residual symptom rate ≤ 50%, a patient's impression that was "improved" or better, and an NRS score ≤ 5) revealed significant and substantial differences in GERD symptoms between these two groups, thereby indicating that the GERD-TEST has a satisfactory concurrent validity.

The GERD-TEST enabled a multifaceted evaluation not only of the severity of symptoms, but also of the impact of the symptoms on daily life, the therapeutic

Table 4 Pearson's *r* value of gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test scores with physical component summary and mental component summary of SF-8 and symptom scores with DS (before treatment; *n* = 290)

	PCS		MCS		Q6. Eating		Q7. Sleeping		Q8. Daily activity		Q9. Mood			
	r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Q1. Heartburn	-0.267	< 0.0001	-0.236	< 0.0001	0.331	< 0.0001	0.420	< 0.0001	0.479	< 0.0001	0.560	< 0.0001	0.558	< 0.0001
Q2. Acid regurgitation	-0.214	0.0003	-0.193	0.0012	0.379	< 0.0001	0.406	< 0.0001	0.458	< 0.0001	0.498	< 0.0001	0.553	< 0.0001
GERD-SS	-0.266	< 0.0001	-0.238	< 0.0001	0.393	< 0.0001	0.457	< 0.0001	0.517	< 0.0001	0.585	< 0.0001	0.614	< 0.0001
Q3. Epigastric pain or burning	-0.311	< 0.0001	-0.327	< 0.0001	0.452	< 0.0001	0.445	< 0.0001	0.483	< 0.0001	0.520	< 0.0001	0.595	< 0.0001
Q4. Postprandial fullness	-0.173	0.0037	-0.402	< 0.0001	0.554	< 0.0001	0.317	< 0.0001	0.448	< 0.0001	0.510	< 0.0001	0.568	< 0.0001
Q5. Early satiation	-0.222	0.0002	-0.354	< 0.0001	0.716	< 0.0001	0.336	< 0.0001	0.440	< 0.0001	0.463	< 0.0001	0.606	< 0.0001
FD-SS	-0.305	< 0.0001	-0.424	< 0.0001	0.658	< 0.0001	0.468	< 0.0001	0.560	< 0.0001	0.611	< 0.0001	0.716	< 0.0001
Q6. Eating	-0.307	< 0.0001	-0.378	< 0.0001										
Q7. Sleeping	-0.216	0.0003	-0.407	< 0.0001										
Q8. Daily activity	-0.356	< 0.0001	-0.494	< 0.0001										
Q9. Mood	-0.307	< 0.0001	-0.496	< 0.0001										
Dissatisfaction for daily life-SS	-0.370	< 0.0001	-0.553	< 0.0001										
	Effect size	Small	Medium	Large										
	r	0.1 ≤	0.3 ≤	0.5 ≤										

GERD-TEST: Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test; PCS: Physical component summary; MCS: Mental component summary; FD: Functional dyspepsia.

response as assessed by the patient. The GERD-TEST is expected to be a useful diagnostic/treatment tool for both clinical research and in daily clinical practice settings, since it consists of relatively few items and subscales that are readily understandable and enable the detection of concurrent FD symptoms.

Symptoms of FD are often seen in patients with GERD[7-11]. The present study results showed that concurrent FD symptoms were noted in as many as 76% of the patients with GERD who met the Montreal definitions, and this finding is consistent with previous reports[7-11]. Symptoms of GERD are generally known to affect various aspects of daily living[5,6], and symptoms of FD have similarly been reported to interfere with the daily living status of patients[13-15], resulting in a reduction in QOL. In the present study, the results of a correlation analysis revealed that both GERD and FD symptoms impair the daily life of patients, affecting eating, sleeping, daily activity and mood (Table 4); these results support those reported by others[5,6,13-15]. Even if a patient presents with a chief complaint of GERD symptoms at the time of their first visit, the possibility that the patient's QOL might be lowered because of concurrent FD and GERD symptoms still exists. Therefore, cases should be carefully selected by observing both FD symptoms and GERD symptoms, and appropriate treatment aimed at treating the former condition should also be administered simultaneously.

Inasmuch as it is often difficult to identify concurrent FD symptoms in patients with GERD, the use of an appropriate PRO might enable such symptoms to not be overlooked, allowing appropriate treatment to proceed. Based on the assumption that GERD and FD are diseases with a spectrum of overlapping symptoms[7-11], the GERD-TEST may allow clinicians to use only one PRO instrument to measure health-related QOL outcomes in patients with GERD, FD, or overlapping symptoms of both conditions.

The use of an appropriate PRO tool for which both reliability and validity have been verified is recommended to ensure evidence-based evaluations of the usefulness of a treatment for disorders such as GERD and FD, where the treatment is primarily aimed at symptomatic improvement[16]. Many PRO tools have been developed and applied in various clinical trials as well as in daily clinical practice settings for the diagnosis of GERD and for evaluating therapeutic responses[18,20]. The practical use and dissemination of PRO as a diagnostic and evaluation tool is anticipated; however, most PROs are lengthy and complicated, and a simple and effective PRO was previously unavailable. The GERD-TEST was developed for this reason.

The goal of treatment for NERD, FD and IBS lies in improving symptoms and signs characteristic of each of these disorders and thereby lessening a patient's sense

Table 5 Comparison of gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores before and after 4 wk of proton pump inhibitor treatment (n = 198)

	Before Tx		After 4 wk PPI Tx		Cohen's d	P value
	mean	SD	mean	SD		
Q1. Heartburn	3.64	1.31	1.77	0.97	1.63	< 0.0001
Q2. Acid regurgitation	3.17	1.37	1.66	0.95	1.29	< 0.0001
GERD-SS	3.40	1.20	1.71	0.91	1.59	< 0.0001
Q3. Epigastric pain or burning	3.11	1.40	1.75	1.02	1.11	< 0.0001
Q4. Postprandial fullness	3.05	1.34	1.95	1.06	0.91	< 0.0001
Q5. Early satiation	2.25	1.34	1.76	0.91	0.42	< 0.0001
FD-SS	2.88	1.13	1.80	0.85	1.08	< 0.0001
Q6. Eating	1.97	1.07	1.41	0.74	0.61	< 0.0001
Q7. Sleeping	2.14	1.07	1.29	0.63	0.97	< 0.0001
Q8. Daily activity	1.98	1.00	1.33	0.67	0.76	< 0.0001
Q9. Mood	2.55	1.06	1.57	0.80	1.05	< 0.0001
Dissatisfaction for daily life-SS	2.15	0.84	1.40	0.59	1.04	< 0.0001
	Effect size	Small	Medium	Large		
	Cohen's d	0.2 ⩽	0.5 ⩽	0.8 ⩽		
	r	0.1 ⩽	0.3 ⩽	0.5 ⩽		

GERD-TEST: Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test; PPI: Proton pump inhibitor; FD: Functional dyspepsia.

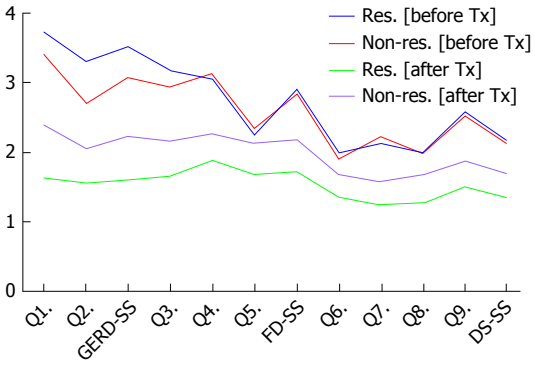

Figure 6 Changes in the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores of responders and non-responders based on a "patient's impression of improved or better" definition at baseline and after 4 wk of proton pump inhibitor therapy. Q1: Heartburn; Q2: Regurgitation; Q3: Epigastric pain/burning; Q4: Postprandial fullness; Q5; Early satiation; Q6: Dissatisfaction with eating; Q7: Dissatisfaction with sleeping; Q8: Dissatisfaction with daily activity; Q9: Dissatisfaction with mood. DS-SS: Dissatisfaction with daily life subscale.

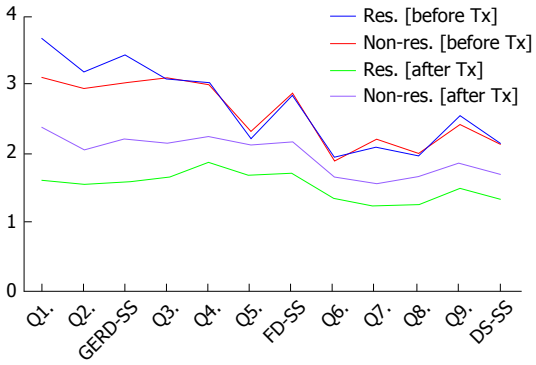

Figure 7 Changes in the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores of responders and non-responders based on a "numeric rating scale ⩽ 5" definition at baseline and after 4 wk of proton pump inhibitor therapy. Q1: Heartburn; Q2: Regurgitation; Q3: Epigastric pain/burning; Q4: Postprandial fullness; Q5: Early satiation; Q6: Dissatisfaction with eating; Q7: Dissatisfaction with sleeping; Q8: Dissatisfaction with daily activity; Q9: Dissatisfaction with mood. DS-SS: Dissatisfaction with daily life subscale.

of burden and impairment of daily living activities. A variety of sets of criteria have been used to evaluate responses to pharmacotherapies for those disorders. Global binary endpoints (a method in which an alternative response to each question is provided, *i.e.*, whether an adequate or satisfactory relief of symptoms has or has not been obtained) and a "residual symptom rate ⩽ 50%" have both exhibited an intense convergent validity and are capable of detecting clinically significant but minimal changes[27]; therefore, these variables are recommended[19,28-30].

A NRS, which is mainly used to evaluate therapeutic responses in patients with chronic pain[31], has been proposed by the FDA as a provisional scale for evaluating abdominal pain in patients with irritable bowel syndrome[32]. An NRS has been recognized as having "higher compliance rates, better responsiveness and ease of use, and good applicability relative to a visual analogue scale".

For evaluating the burden by the symptoms as well as the response to the therapy, the GERD-TEST can be applied using three definitions: *i.e.*, a 7-point Likert scale for individual symptoms, the patient's impression of the therapy (which corresponds to the OTE), and the NRS (as recommended by various reports and guidelines), and interestingly, the global assessments of the GERD symptoms using patient's impression of the therapy (Q11) and NRS (Q12) well differentiated from those of FD symptoms (Table 9). Therefore, evaluations of patient burden arising from various symptoms and of the comprehensive therapeutic response using this tool are thought to be appropriate.

Of the plurality of therapeutic response evaluation definitions currently available, none have been shown to be optimal for the evaluation of therapeutic responses during the management of GERD. It is thus

Table 6 Comparison of the gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores between responders and non-responders based on a "residual symptom rate ⩽ 50%" definition

Responder definition by	Before Tx						After 4 wk PPI Tx						⊿ (0W-4W)					
	Responder (*n* = 153)		Non-responder (*n* = 41)		Cohen's *d*	*P* value	Responder (*n* = 153)		Non-responder (*n* = 41)		Cohen's *d*	*P* value	Responder (*n* = 153)		Non-responder (*n* = 41)		Cohen's *d*	*P* value
Residual Sx 50% ⩾	mean	SD	mean	SD			mean	SD	mean	SD			mean	SD	mean	SD		
Q1. Heartburn	3.79	1.29	3.34	1.09	0.36	0.025	1.45	0.61	3.05	1.07	2.21	< 0.0001	-2.34	1.24	-0.29	0.87	1.76	< 0.0001
Q2. Acid regurgitation	3.31	1.32	2.85	1.37	0.34	0.060	1.37	0.57	2.78	1.24	1.86	< 0.0001	-1.93	1.25	-0.07	1.17	1.51	< 0.0001
GERD-SS	3.55	1.19	3.10	0.99	0.39	0.014	1.41	0.53	2.91	1.08	2.21	< 0.0001	-2.14	1.11	-0.18	0.71	1.89	< 0.0001
Q3. Epigastric pain or burning	3.12	1.42	3.27	1.18	-	0.509	1.51	0.84	2.73	1.10	1.36	< 0.0001	-1.61	1.41	-0.54	1.03	0.81	< 0.0001
Q4. Postprandial fullness	3.05	1.39	3.15	1.15	-	0.636	1.79	0.96	2.61	1.18	0.81	< 0.0001	-1.25	1.32	-0.54	1.16	0.56	0.001
Q5. Early satiation	2.26	1.38	2.28	1.20	-	0.950	1.66	0.85	2.17	1.05	0.57	0.004	-0.60	1.13	-0.10	0.90	0.46	0.003
FD-SS	2.89	1.17	2.98	0.94	-	0.622	1.62	0.74	2.56	0.85	1.24	< 0.0001	-1.27	1.06	-0.42	0.82	0.84	< 0.0001
Q6. Eating	1.95	1.05	2.07	1.15	-	0.550	1.34	0.69	1.68	0.88	0.47	0.023	-0.61	0.88	-0.39	1.02	0.25	0.206
Q7. Sleeping	2.17	1.08	2.05	1.05	-	0.514	1.18	0.49	1.66	0.88	0.80	0.001	-0.99	1.02	-0.39	0.86	0.61	0.000
Q8. Daily activity	1.99	1.03	1.98	0.89	-	0.909	1.24	0.6	1.68	0.82	0.68	0.001	-0.75	1.05	-0.30	0.85	0.45	0.005
Q9. Mood	2.60	1.07	2.46	0.98	-	0.433	1.43	0.74	2.10	0.83	0.88	< 0.0001	-1.17	1.06	-0.37	0.80	0.80	< 0.0001
Dissatisfaction for daily life-SS	2.18	0.83	2.11	0.84	-	0.650	1.30	0.52	1.78	0.69	0.86	< 0.0001	-0.88	0.77	-0.36	0.64	0.71	< 0.0001
	Effect size	Small	Medium	Large														
	Cohen's *d*	0.2 ⩽	0.5 ⩽	0.8 ⩽														

GERD-TEST: Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test; PPI: Proton pump inhibitor.

considered preferable to report data obtained and analyzed using two or more therapeutic response evaluation definitions, rather than any single definition.

The limitations of this study were, firstly, the clinical responses in terms of the GERD symptoms can be evaluated using three definitions in the GERD-TEST; these definitions were formulated chiefly for the diagnosis and treatment of GERD. Concurrent FD symptoms, however, can only be evaluated using a residual symptom rate. The GERD-TEST should be modified to include the patient's impression and NRS items for FD symptom, similar to the GERD symptom evaluations, to make this definition even more useful for the diagnosis and treatment of FD. Secondly, it is generally recognized that patients with GERD or FD present with diverse symptoms. Among patients with GERD, non-typical symptoms such as esophageal symptoms (*e.g.*, chest pain) and extraesophageal symptoms (*e.g.*, chronic cough, chronic laryngitis, asthma or dental erosion[1] are often seen. Symptoms such as bloating, belching or nausea develop among patients with FD. Clinical evaluation using the GERD-TEST is focused primarily on the cardinal symptoms of GERD and FD, and the evaluation does not cover patient burden from other symptoms or the impacts of such symptoms on daily life. Further investigation and clarification of these matters is also needed.

In conclusion, the psychometric characteristics of the GERD-TEST were excellent, demonstrating good validity and reliability. The GERD-TEST is simple and easy to perform and is a multifaceted PRO instrument that appears to be useful for evaluating disease-specific health-related QOL in GERD patients in both clinical trial and primary care settings.

ACKNOWLEDGMENTS

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Table 7 Comparison of gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores between responders and non-responders based on a "patient's impression of improved or better" definition

Responder definition by	Before Tx						After 4 wk PPI Tx						⊿ (0W-4W)					
	Responder (*n* = 155)		Non-responder (*n* = 39)		Cohen's *d*	*P* value	Responder (*n* = 155)		Non-responder (*n* = 39)		Cohen's *d*	*P* value	Responder (*n* = 155)		Non-responder (*n* = 39)		Cohen's *d*	*P* value
Patients' impression improved ⩽	mean	SD	mean	SD			mean	SD	mean	SD			mean	SD	mean	SD		
Q1. Heartburn	3.72	1.31	3.41	1.25	-	0.169	1.62	0.88	2.38	1.14	0.82	0.000	-2.10	1.42	-1.03	1.25	0.78	< 0.0001
Q2. Acid regurgitation	3.30	1.39	2.69	1.20	0.45	0.007	1.55	0.86	2.05	1.19	0.53	0.015	-1.74	1.39	-0.64	1.44	0.79	< 0.0001
GERD-SS	3.51	1.23	3.05	1.01	0.39	0.017	1.59	0.83	2.22	1.08	0.71	0.001	-1.92	1.29	-0.83	1.10	0.87	< 0.0001
Q3. Epigastric pain or burning	3.17	1.42	2.92	1.31	-	0.306	1.65	0.98	2.15	1.16	0.50	0.014	-1.52	1.45	-0.77	1.11	0.54	0.001
Q4. Postprandial fullness	3.04	1.39	3.13	1.22	-	0.691	1.88	1.07	2.26	1.04	0.36	0.045	-1.16	1.33	-0.87	1.28	0.22	0.212
Q5. Early satiation	2.23	1.38	2.33	1.24	-	0.662	1.67	0.88	2.13	0.98	0.51	0.008	-0.56	1.13	-0.21	0.95	0.33	0.044
FD-SS	2.90	1.17	2.83	0.97	-	0.697	1.71	0.84	2.17	0.83	0.55	0.002	-1.19	1.10	-0.65	0.86	0.51	0.001
Q6. Eating	1.99	1.08	1.90	1.07	-	0.641	1.35	0.70	1.67	0.87	0.43	0.036	-0.64	0.89	-0.23	0.90	0.46	0.013
Q7. Sleeping	2.12	1.08	2.21	1.08	-	0.646	1.23	0.58	1.56	0.79	0.55	0.013	-0.89	1.04	-0.64	0.93	0.25	0.146
Q8. Daily activity	1.98	1.01	1.97	0.97	-	0.968	1.26	0.63	1.67	0.74	0.63	0.002	-0.72	1.02	-0.32	0.96	0.41	0.021
Q9. Mood	2.57	1.07	2.51	1.05	-	0.771	1.50	0.8	1.87	0.77	0.47	0.007	-1.07	1.06	-0.64	1.01	0.41	0.020
Dissatisfaction for daily life-SS	2.16	0.84	2.12	0.84	-	0.768	1.33	0.57	1.69	0.63	0.62	0.001	-0.83	0.77	-0.45	0.68	0.50	0.003
	Effect size	Small	Medium	Large														
	Cohen's *d*	0.2 ⩽	0.5 ⩽	0.8 ⩽														

GERD-TEST: Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test; PPI: Proton pump inhibitor.

Table 8 Comparison of gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test scores between responders and non-responders based on a "numeric rating scale ⩽ 5" definition

Responder definition by	Before Tx						After 4 wk PPI Tx						⊿ (0W-4W)					
	Responder (*n* = 176)		Non-responder (*n* = 19)		Cohen's *d*	*P* value	Responder (*n* = 176)		Non-responder (n = 19)		Cohen's *d*	*P* value	Responder (*n* = 176)		Non-responder (*n* = 19)		Cohen's *d*	*P* value
NRS 5 ⩾	mean	SD	mean	SD			mean	SD	mean	SD			mean	SD	mean	SD		
Q1. Heartburn	3.68	1.32	3.11	0.99	0.45	0.022	1.69	0.89	2.37	1.30	0.73	0.027	-1.99	1.42	-0.74	1.15	0.90	< 0.0001
Q2. Acid regurgitation	3.19	1.37	2.95	1.22	-	0.423	1.56	0.84	2.32	1.16	0.87	0.006	-1.63	1.39	-0.63	0.83	0.75	< 0.0001
GERD-SS	3.43	1.21	3.03	1.02	-	0.106	1.62	0.81	2.34	1.21	0.85	0.012	-1.81	1.27	-0.68	0.87	0.91	< 0.0001
Q3. Epigastric pain or burning	3.08	1.38	3.11	1.41	-	0.940	1.66	0.95	2.37	1.21	0.73	0.014	-1.42	1.40	-0.74	1.37	0.49	0.040
Q4. Postprandial fullness	3.04	1.35	3.00	1.25	-	0.896	1.90	1.05	2.42	1.12	0.50	0.053	-1.14	1.32	-0.58	1.17	0.43	0.051
Q5. Early satiation	2.22	1.33	2.32	1.29	-	0.753	1.69	0.89	2.26	0.93	0.64	0.012	-0.53	1.06	-0.05	1.03	0.45	0.058
FD-SS	2.85	1.13	2.88	1.02	-	0.899	1.73	0.82	2.36	0.82	0.77	0.002	-1.13	1.08	-0.53	0.83	0.57	0.004
Q6. Eating	1.94	1.05	1.89	0.99	-	0.841	1.35	0.69	1.63	0.68	0.40	0.095	-0.59	0.92	-0.26	0.81	0.36	0.101
Q7. Sleeping	2.10	1.05	2.21	1.08	-	0.679	1.23	0.55	1.84	1.01	1.02	0.010	-0.88	1.00	-0.37	0.90	0.51	0.022
Q8. Daily activity	1.97	1.01	2.00	0.94	-	0.881	1.26	0.59	1.95	0.91	1.10	0.001	-0.71	1.04	-0.05	0.40	0.67	< 0.0001
Q9. Mood	2.55	1.08	2.42	0.84	-	0.534	1.51	0.78	2.05	0.85	0.70	0.008	-1.05	1.08	-0.37	0.50	0.65	< 0.0001
Dissatisfaction for daily life-SS	2.13	0.83	2.13	0.84	-	0.989	1.34	0.53	1.87	0.77	0.95	0.004	-0.81	0.78	-0.26	0.43	0.73	< 0.0001
	Effect size	Small	Medium	Large														
	Cohen's *d*	0.2 ⩽	0.5 ⩽	0.8 ⩽														

GERD-TEST: Gastroesophageal reflux and dyspepsia-therapeutic efficacy and satisfaction test; PPI: Proton pump inhibitor.

Table 9 Type of symptoms responding to the therapeutic efficacy as assessed by the patients (Multivariate analysis)

	Q11. Patient's impression		Q12. Numeric rating scale	
	β	*P* value	β	*P* value
ΔGERD-SS (0-4 wk)	0.371	< 0.0001	0.411	< 0.0001
ΔFD-SS (0-4 wk)	0.037	0.6541	-0.092	0.2656
R^2 (*P* value)	0.155	< 0.0001	0.133	< 0.0001
	Effect size	Small	Medium	Large
	β	0.1 ≤	0.3 ≤	0.5 ≤
	R^2	0.02 ≤	0.13 ≤	0.26 ≤

Nobuyuki Matsuhashi, NTT Medical Center Tokyo; Mineo Kudo, Sapporo Hokuyu Hospital; Norimasa Yoshida and Takahiro Suzuki, Japanese Red Cross Kyoto Daiichi Hospital; Kazunari Murakami and Seiji Shiota, Oita University Faculty of Medicine; Mototsugu Kato and Katsuhiro Mabe, Hokkaido University Hospital; Tsuyoshi Sanuki and Junko Hori, Kita Harima Medical Center; Noriaki Manabe and Ken Haruma, Kawasaki Medical School; Yuji Naito and Osamu Handa, Kyoto Prefectural University of Medicine; Syuuji Inoue, National Hospital Organization Kochi Hospital; Hirokazu Oyamada and Yutaka Isozaki, Matsushita Memorial Hospital; Shuichi Muto, Tomakomai City Hospital; Kenji Furuta and Shunji Ohara, Shimane University Faculty of Medicine; Tadayuki Oshima and Hiroto Miwa, Hyogo College of Medicine; Yasuhiro Fujiwara and Yukie Kohata, Osaka City University Graduate School of Medicine; Kazuhiro Maeda and Yuji Sakai, Tenjin Clinic, Medical Corporation Shin-ai; Yugo Iwaya and Sadahisa Okuhara, Shinshu University School of Medicine; Takashi Abe and Yongmin Kim, Takarazuka Municipal Hospital; Hideki Mizuno, Toyama City Hospital; Kimio Isshi, Isshi Gastro-Intestinal Clinic; Hiroshi Seno and Tsutomu Chiba, Kyoto University Hospital; Fukunori Kinjo and Manabu Nakamoto, University Hospital, University of the Ryukyus; Toshiro Sugiyama and Haruka Fujinami, University of Toyama; Keiko Utsumi, Aichi Medical University Medical Clinic; Hiroyuki Kuwano and Tatsuya Miyazaki, Gunma University Hospital; Noriko Watanabe, National Hospital Organization Mie Chuo Medical Center; Fumihiko Kinekawa and Kita Yuko, Sanuki Municipal Hospital; Tomonori Imaoka and Hirohumi Fujishiro, Shimane Prefectural Central Hospital; Takatsugu Yamamoto and Yasushi Kuyama, Teikyo University School of Medicine; Yasuaki Nakajima and Kenro Kawada, Tokyo Medical and Dental University.

COMMENTS

Background

The use of an appropriate patient-reported outcome (PRO) instrument may facilitate the detection of gastroesophageal reflux disease (GERD) patients, and the evaluation of the disease's impact on daily life and the response to the therapy. However, a simple and effective PRO was previously unavailable.

Research frontiers

The importance of PRO in evaluating medical care has been stressed in recent years. The Food and Drug Administration guidance recommends the use of valid and appropriate PRO for each disease.

Innovations and breakthroughs

Most of the previously developed PROs for GERD were lengthy and complicated, and even not well validated. The lack of a simple, easy to understand instrument for GERD patients encouraged the development of the gastroesophageal reflux and dyspepsia therapeutic efficacy and satisfaction test (GERD-TEST). The GERD-TEST minimized the number of items and enabled a multifaceted evaluation not only of the severity of symptoms, but also of the impact of the symptoms on daily life and of the therapeutic response as assessed by the patient. The psychometric characteristics of the GERD-TEST were excellent, demonstrating good validity and reliability.

Applications

This study indicated that the GERD-TEST is a useful tool for the clinical research in GERD patients. Since the GERD-TEST is simple and easy-to-understand, which also could applied for daily clinical practice settings.

Terminology

The PRO instrument with proven reliability and validity is useful for the disorders in which the treatment goal is to ameliorate symptoms. The application of PRO not only in clinical trials, but also in daily clinical practice settings would enable greater objectivity in the diagnosis and evaluation of therapeutic responses in GERD cases and the provision of effective and efficient treatment.

Peer-review

The study is well done and the methodology is strong. The clinical meaning is also relevant, because the authors have addressed the frequent overlap between esophageal and dyspepsia symptoms. This investigation merits to be published.

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ORIGINAL ARTICLE

Observational Study

Modified B-ultrasound method for measurement of antral section only to assess gastric function and guide enteral nutrition in critically ill patients

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Abstract

AIM

To establish a modified B-ultrasound method of measuring the antral section only to assess gastric motility in healthy people, and evaluate its application in guiding enteral nutrition (EN) in critically ill patients.

METHODS

First, 30 healthy volunteers were selected. The modified B-ultrasound method and the traditional B-ultrasound method were applied to assess gastric function. The correlation of indices of gastric function between the two groups was analyzed statistically. In addition, 64 critically ill patients were selected, and the modified B-ultrasound method and the gastric juice withdrawal method were applied to guide the implementation of EN. Daily caloric value, the time required to achieve complete EN, ICU stay, hospitalization time, and serum prealbumin and albumin levels were recorded and compared between the two groups. Kaplan-Meier survival curve was used to compare the complications of EN between the two groups.

RESULTS

In healthy subjects, there was a good correlation among gastric emptying time, antral contraction frequency and

antral motility index between the two groups (r = 0.57, 0.61 and 0.54, respectively). The study on critically ill patients also revealed that a better effect of EN was achieved in the modified B-ultrasound method group, in which patients had shorter ICU stay and hospitalization time and higher levels of serum prealbumin and albumin. The Kaplan-Meier survival analysis revealed that the improved B-ultrasound method was associated with significantly fewer EN complications (P = 0.031).

CONCLUSION

The modified B-ultrasound method can provide a good real-time assessment of gastric function and has a better effect than the traditional method in guiding EN in critically ill patients.

Key words: Gastric emptying; Real-time ultrasound; Critically ill patients; Enteral nutrition

Core tip: In order to provide critically ill patients with timely postoperative enteral nutrition (EN), a modified B-ultrasound method for measurement of antral section only was established. In healthy subjects, there was a good correlation among gastric emptying time, antral contraction frequency and antral motility index between the modified and traditional methods. The study on critically ill patients also revealed that a better effect of EN was achieved in the modified B-ultrasound method group with regard to patients' hospitalization conditions and the incidence of EN complications.

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INTRODUCTION

Critically ill patients with postoperative stress response and multisystem disorders have an increased risk of infection and even death. Timely postoperative enteral nutrition (EN) support can significantly improve the prognosis of these patients[1-6]. However, critically ill patients often have varying degrees of gastrointestinal motility disorders and other disorders. Therefore, individualized EN programs should be developed for patients with varying degrees of gastrointestinal dysfunction[7-10]. In clinical practice, medical staff often uses retractable gastric tubes to withdraw residual gastric juice to assess patients' gastric motility and guide EN. Due to the miniaturization and portability of B-ultrasound equipment, some medical institutions also use B-ultrasound to measure the antral section only for real-time guidance of EN[11,12]. Accurate measurement of residual gastric volume is very important for subsequent EN. On one hand, the gastric tube will be inserted too shallowly if the measured residual gastric volume is too small. This will cause the EN rate to be excessively high, which can cause bloating, reflux, pneumonia and other complications. On the other hand, the gastric tube will be inserted too deeply when the measured residual gastric volume is too large. This would easily lead to gastric mucosal injury during operation. Traditional B-ultrasound measurement of the antral section only requires patients to maintain the standing position while drinking 500 mL of liquid. However, critically ill patients often have difficulties tolerating such position[13,14]. Therefore, this study aimed to improve the traditional B-ultrasound method by placing patients in the semirecumbent position to fill the gastric cavity with 300 mL of ultrasound solution, which is followed by real-time monitoring of gastrointestinal motility, with an aim to provide individualized EN programs for critically ill patients.

MATERIALS AND METHODS

Inclusion and exclusion criteria

The inclusion criteria were: (1) critically ill patients (52 cases) admitted in the intensive care unit of our hospital from January 2014 to December 2015; (2) patients with an APACHE II score $\geqslant$ 8; and (3) patients in a stable condition during the recovery period. The exclusion criteria were: (1) patients who underwent gastric resection; (2) patients with flatulence that could not be observed; and (3) patients who were not suitable for gastrointestinal perfusion due to various reasons. At the same time, 30 healthy subjects were recruited to evaluate the feasibility of the modified B-ultrasound method in assessing gastrointestinal function. This study was approved by the Ethics Committee of Cangzhou Central Hospital. All subjects provided a signed informed consent form.

Evaluation of the feasibility of the modified B-ultrasound method in monitoring gastric function

Assessment of gastric emptying: The SonoSite TITAN portable B-ultrasound machine (Bothell, WA, United States) was used for assessing gastric function in 30 healthy subjects. Indexes evaluated included: fasting antral area, the immediate maximum antral relaxation area after filling the stomach, 5-min changes in the antral diastolic area until the liquid dark area disappeared after filling the stomach, the disappearance time of the liquid dark area in the stomach after filling (gastric emptying time, GET), 5-min antral contractions after filling, and three consecutive maximum antral relaxation and contraction areas ($S_{relaxation}$ and $S_{contraction}$). Based on the above data, the following were calculated: antral area changes (ΔS) = $S_{relaxation}$ - $S_{contraction}$; antral contraction

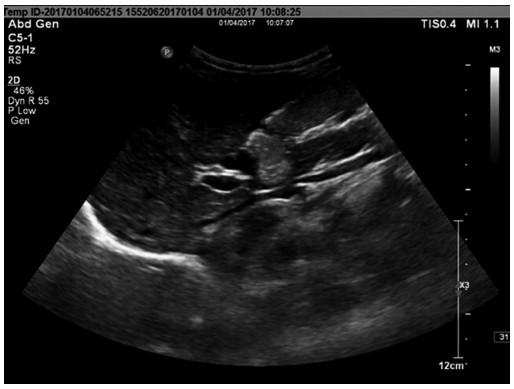

Figure 1 Ultrasound measurement of the antral section only.

frequency (ACF) = the number of antral contractions within 5 min after gastric filling/5; antral contraction amplitude (ACA) = $\Delta S/S_{relaxation}$; antral motility index (MI) = ACF × ACA.

Detection method: The subjects were fasted for 8 h overnight, and on the next morning, the traditional B-ultrasound method was used to assess gastric function. Briefly, the subjects were placed in the supine position and instructed to drink 500 mL of ultrasound liquid within 2 min, and gastric indexes were then detected after gastric filling. The subjects were then fasted again for 8 h overnight, and on the morning of the next day, gastric function was assessed using the improved B-ultrasound method, in which the subjects were instructed to drink 300 mL of ultrasound liquid within 2 min in the semirecumbent position, and gastric indexes were then detected after gastric filling.

Comparison of the effects of the modified B-ultrasound method and the gastric juice withdrawal method in guiding EN for critically ill patients

Assessment of gastric function in critically ill patients using the gastric juice withdrawal method: The distance from the hairline to the sternum of patients was used as the length of the indwelling gastric tube (the size was approximately 45-55 cm). Gastric remnants were measured by artificial aspiration every 50 h using a 50-mL syringe. According to the American Society for Parenteral and Enteral Nutrition guidelines on EN, the EN program was developed as follows: the target feeding amount was set at 104.6-125.5 KJ (25 to 30 kcal)/(kg•d); the EN rate was adjusted according to the patient's MI, and the gastric function of patients was detected every one hour. If MI was < 0.4, the EN rate was set at 20-30 mL/h; when MI was ⩾ 0.4 but < 0.8, the EN rate was set at 40-60 mL/h; when MI was ⩾ 0.8, the EN rate was set at ⩾ 70 mL/h.

It has been believed that full EN can be achieved when the target feeding amount reaches > 80%. During the EN process, the tolerance of patients should be closely monitored. The incidence of adverse complications within 10 d after the initiation of EN was recorded (reflux, vomiting, diarrhea, abdominal distension, and new-onset pneumonia).

Research method: According to the severity of disease, 64 critically ill patients were selected and divided into two groups (n = 32 each), and the modified B-ultrasound method and the gastric juice withdrawal method were, respectively, applied to monitor gastric function and guide the implementation of EN. The following EN indexes were collected and recorded: EN start time, the time required to reach the maximum feeding rate, EN-related complications (including reflux, new-onset pneumonia, vomiting, diarrhea, abdominal distension, *etc*.) and the onset time, and changes in serum prealbumin and albumin.

Statistical analysis

Statistical analyses were performed using SPSS20.0 software package. Normally distributed variables are expressed as mean ± SD, and non-normally distributed data are expressed as median (interquartile range). Data on gastric function of the healthy subjects were analyzed using paired t-test and linear correlation analysis. The two groups of critically ill patients were compared using the two independent samples t-test. Kaplan-Meier survival curves were used to compare differences in the complications of EN in the two groups. $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of gastric function using the modified B-ultrasound method

The antral section was gradually detected on the abdominal surface after the ultrasound imaging revealed the superior mesenteric vein, abdominal aorta and the left lobe of the liver. This test point was used as a marker of the antral section (Figure 1) to detect related indexes. Gastric function indices were detected using the two types of B-ultrasound methods. As shown in Table 1, ACF, ACA, MI, GET and other indexes detected using the modified B-ultrasound method were significantly smaller than those detected using the traditional B-ultrasound method ($P < 0.05$ for all). Linear correlation analysis showed that ACF, ACA and GET detected using the traditional method were highly correlated with those detected using the modified method, although there was a poor correlation for ACA (Table 2).

Comparison of the effects of the modified B-ultrasound method and the gastric juice withdrawal method in guiding EN

The effects of the two methods in guiding EN are shown in Table 3. The modified B-ultrasound method had a better effect in guiding EN. EN duration, EN

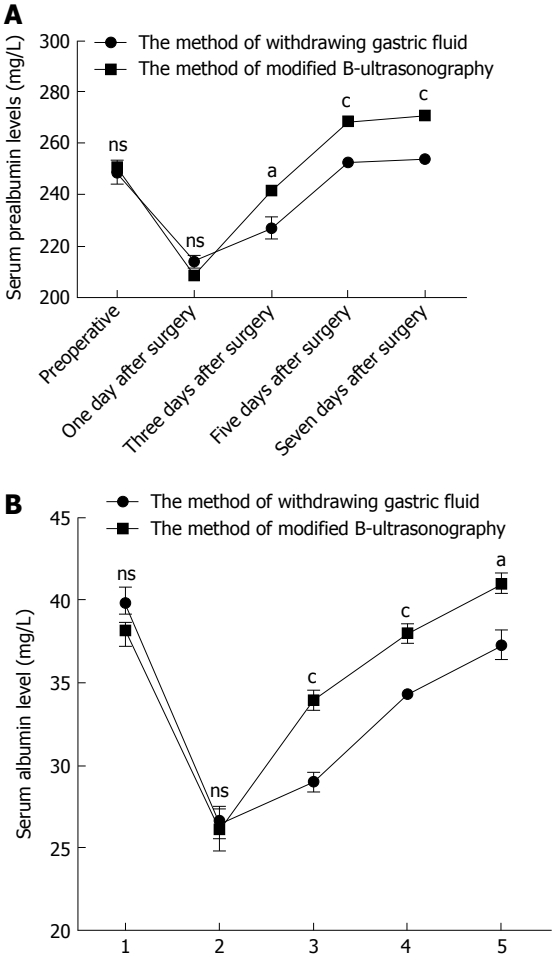

Figure 2 Comparison of changes in prealbumin and albumin levels between the two groups of patients. A: Changes in prealbumin levels; B: Changes in albumin levels. $^{a}P < 0.05$, $^{c}P < 0.01$.

compliance time, and average daily calorie value were significantly higher in the modified B-ultrasound method group than in the gastric juice withdrawal group ($P < 0.05$ for all). Prealbumin and albumin levels on postoperative days 3, 5 and 7 were also significantly higher in the modified B-ultrasound method group than in the gastric juice withdrawal group ($P < 0.05$ for all) (Figures 2 and 3). Kaplan-Meier survival analysis revealed that the incidence of complications (reflux, vomiting, diarrhea, abdominal distension, and new-onset pneumonia) was significantly lower in the modified B-ultrasound method group than in the gastric juice withdrawal group ($P = 0.031$).

DISCUSSION

In both patients and healthy people, adequate intake of nutrients is important for maintaining the normal function of the body, and good nutrition helps to maintain cell metabolism, the normal structure of tissues and organs, and other functions[15-19]. At present, parenteral nutrition, usually through the intestine, provides patients with good nutritional support. The EN approach has gained the attention of clinicians, because this approach is closer to the normal physiological conditions of the body's intake of nutrients[20-25]. However, for patients suffering from severe diseases, their gastrointestinal function may be impaired in varying degrees, which mainly leads to gastric emptying disorders and EN intolerance[26-33]. Therefore, it is important to monitor their gastric function in real time, in order to develop an individualized EN regimen for patient rehabilitation. In this study, our results showed that, in the normal population, the modified B-ultrasound method can also provide a good reflection of the gastric function. On this basis, its practical application in critically ill patients also suggests that the modified B-ultrasound method can guide the EN program well for patients requiring nutritional support and reduce the incidence of EN complications.

Table 1 Comparison of the indices of gastric function measured using the modified and traditional B-ultrasound methods

Index	Modified method (n = 32)	Traditional method (n = 32)	t-value	P value
ACF	2.39 ± 0.24	3.22 ± 0.32	-10.580	0.000
ACA	0.36 ± 0.04	0.69 ± 0.11	-14.376	0.000
MI	3.34 ± 0.25	4.37 ± 0.34	-12.445	0.000
GET	32.65 ± 4.46	60.44 ± 4.98	-21.196	0.000

ACF: Antral contraction frequency; ACA: Antral contraction amplitude; MI: Motility index; GET: Gastric emptying time.

Table 2 Correlation analysis of indices measured using the modified and traditional methods

Index	Correlation coefficient	P value	95%CI
ACF	0.613	0.003	0.21-0.74
ACA	0.324	0.080	-0.06-0.63
MI	0.536	0.005	0.19-0.76
GET	0.572	0.004	0.21-0.77

ACF: Antral contraction frequency; ACA: Antral contraction amplitude; MI: Motility index; GET: Gastric emptying time.

Evaluation of the feasibility of the modified B-ultrasound method in assessing gastric function

B-ultrasound has been widely used in various departments due to its simple, convenient, accurate and reproducible features. In 1989, Marzio*etal*[14] first proposed to use B-ultrasound to measure the antrum only for real-time monitoring of gastric emptying, in order to assess gastric function. However, this method requires the patient to maintain an upright posture while drinking 500 mL liquid. Hence, this method cannot be tolerated by many critically ill patients due to gastric dysfunction and other reasons[14]. Therefore, we improved this traditional method in this study by placing patients in the semirecumbent position while drinking 300 mL of ultrasound solution to fill the antrum. Then, guided EN was performed after portable B-ultrasound equipment was used to

Table 3 Comparison of the implementation of enteral nutrition in the two groups of patients

	ICU time (d)	Hospitalization time (d)	Enteral nutrition time (h)	Average daily calorie value (kcal/kg)
Modified B-ultrasound method (n = 26)	4.42 ± 1.92	13.35 ± 2.92	30.38 ± 9.42	28.73 ± 4.35
Gastric juice withdrawal method (n = 26)	5.31 ± 2.11	16.58 ± 5.95	36.63 ± 10.26	25.69 ± 3.74
t-value	-1.620	-2.482	-2.123	2.702
P value	0.111	0.016	0.039	0.009

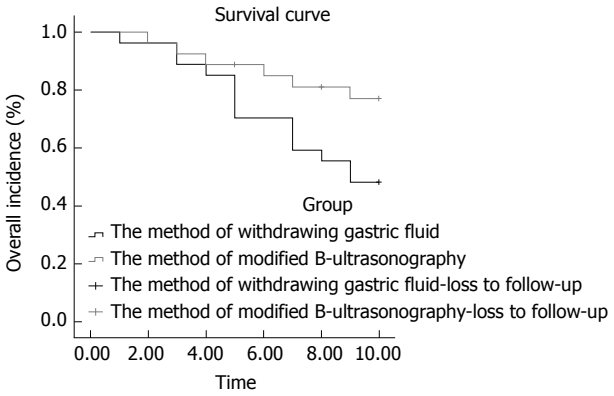

Figure 3 Comparison of complications between the two groups of patients.

detect relevant indexes of gastric function in patients. The differences in and correlations of various gastric function indexes between the modified and traditional methods were investigated in a normal population using a self-controlled method to verify the reliability of the improved B-ultrasound method in assessing gastric function.

Stomach contents can stimulate gastric emptying, and the mechanical stimulation of the vagus nerve reflex in the gastric wall increases stomach movement. In general, the rate of gastric emptying is proportional to the amount of stomach contents[34-43]. In the present study, the difference in gastric wall nerve stimulation was caused by the different volumes of ultrasound liquid. The indexes (ACA, ACF, MI and GET) detected using the modified B-ultrasound method were significantly smaller than those obtained using the conventional method. Since the rate of gastric emptying is regulated by nerves, body fluids and a variety of factors, ACA derived by these two detection methods had a poor correlation. However, the rest important indexes (ACF, GET and MI) are highly correlated between the two methods. This suggests that the modified B-ultrasound method has the potential to enable the real-time detection of gastric function.

Comparison of the effects of the modified B-ultrasound method and the gastric juice withdrawal method in guiding EN

The self-controlled study of healthy people revealed that the modified B-ultrasound method has a potential to detect gastric function. We therefore further investigated the effects of the modified B-ultrasound method and the gastric fluid withdrawal method in guiding EN in critically ill patients.

The gastric juice withdrawal method is a method of guiding EN by detecting gastric residues, which has been widely used in many hospitals[44-51]. However, a variety of factors including the length and location of the indwelling tube would result in the lack of accuracy of the test results, and some deficiencies often occur in the implementation of this method for guiding EN. If the indwelling tube is inserted too shallowly, this causes the amount of residual calorie measurement to be small and would increase EN speed, which often results in bloating, reflux, new-onset pneumonia and other complications. If the indwelling tube is placed too deeply, the measured residual gastric volume will be excessively large, which often causes gastric mucosal damage. Hence, there is an urgent need for a more effective method for clinical applications.

When comparing the effects of the two methods in guiding EN, the average calorie intake of patients receiving EN was significantly higher when the modified B-ultrasound method was used. Furthermore, the time required to achieve complete EN was also significantly less for the modified B-ultrasound method. As a result, patients who used the modified B-ultrasound method obtained better nutritional support. In addition, the modified B-ultrasound method exhibited a significant advantage in reflecting the nutritional status of patients with regard to plasma prealbumin and albumin levels and other indexes. The recovery rates of prealbumin and albumin levels within 7 d after surgery were higher in patients of the modified B-ultrasound method group than in the traditional gastric juice withdrawal group. According to a multicenter survey that involved 26 hospitals in Europe, the nutritional status of patients was significantly negatively correlated with hospital stay, complications and mortality, and patients who received good nutritional support had shorter hospital stay, fewer complications and increased survival rate[52-55]. In the present study, the Kaplan-Meier survival analysis revealed that the complications of patients with good nutritional support were significantly fewer than those in the traditional gastric juice withdrawal group. Furthermore, the durations of ICU stay and hospital stay in the modified B-ultrasound method group were also significantly shorter than those in the traditional method group. The underlying reason for this is that patients had a better recovery in the modified B-ultrasound method group, because the modified method can more accurately assess the gastric function of patients, and is conducive for clinicians to accurately

control the EN speed. As a result, the incidence of vomiting, bloating, new-onset pneumonia and other complications was also significantly lower. These results suggest that the modified B-ultrasound method is an effective method for guiding EN.

However, since the B-ultrasound method is susceptible to stomach gas interference, B-ultrasound detection was not performed in our three patients due to severe flatulence. Therefore, future studies should be required to further evaluate the types of disease suitable for B-ultrasound detection.

In summary, the modified B-ultrasound method can better assess gastric function and guide EN in critically ill patients than the traditional gastric juice withdrawal method, showing good clinical value.

COMMENTS

Background

Enteral nutrition (EN) is widely used widely in clinical practice for the reason that this approach is closer to the normal physiological conditions of the body's intake of nutrients. However, since critically ill patients often suffer from varying degrees of gastric dysfunction and have EN intolerance, it is important to monitor their gastric function in real time, in order to develop an individualized EN regimen for patient rehabilitation.

Research frontiers

In clinical practice, medical staff often uses retractable gastric tubes to withdraw residual gastric juice to assess patients' gastric motility and guide EN. Due to the miniaturization and portability of B-ultrasound equipment, some medical institutions also use B-ultrasound to measure the antral section only for real-time guidance of EN. Given the fact that this method has some deficiencies, more methods should be developed to provide crucially ill patients with EN in real time.

Innovations and breakthroughs

In this study, a modified B-ultrasound method for measuring the antral section only was established. In healthy subjects, there was a good correlation among gastric emptying time, antral contraction frequency and antral motility index between the modified and normal methods. When guided by the modified method, the study on critically ill patients also revealed that a better effect of EN was achieved with regard to patients' hospitalization conditions and the incidence of EN complications.

Applications

The modified method that has a better effect in providing guidance of EN can better provide nutritional support for critically ill patients, and contribute to the rehabilitation of patients.

Peer-review

This is an interesting study about the establishment of a modified B-ultrasound method for measurement of the antral section only and its application value in guiding EN in critically ill patients.

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ORIGINAL ARTICLE

Prospective Study

Chronic liver failure-consortium acute-on-chronic liver failure and acute decompensation scores predict mortality in Brazilian cirrhotic patients

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Abstract

AIM

To validate prognostic scores for acute decompensation of cirrhosis and acute-on-chronic liver failure in Brazilian patients.

METHODS

This is a prospective cohort study designed to assess the prognostic performance of the chronic liver failure-consortium (CLIF-C) acute decompensation score (CLIF-C AD) and CLIF-C acute-on-chronic liver failure score (CLIF-C ACLF), regarding 28-d and 90-d mortality, as well as to compare them to other prognostic models, such as Model for End-Stage Liver Disease (MELD), MELD Sodium (MELD-Na), Child-Pugh (CP) score, and the CLIF-C Organ Failure score (CLIF-C OF). All participants were adults with acute decompensation of cirrhosis admitted to the Emergency Department of a tertiary hospital in southern Brazil. Prognostic performances were evaluated by means of the receiver operating characteristic (ROC) curves, area

under the curves (AUC) and 95%CI.

RESULTS

One hundred and thirteen cirrhotic patients were included. At admission, 18 patients had acute-on-chronic liver failure (ACLF) and 95 individuals had acute decompensation (AD) without ACLF, of which 24 eventually developed ACLF during the course of hospitalization (AD evolving to ACLF group). The AD group had significantly lower 28-d (9.0%) and 90-d (18.3%) mortality as compared to the AD evolving to ACLF group and to the ACLF group (both $P < 0.001$). On the other hand, 28-d and 90-d mortalities were not significantly different between AD evolving to ACLF group and ACLF group ($P = 0.542$ and $P = 0.708$, respectively). Among patients with ACLF, at 28 d from the diagnosis, CLIF-C ACLF was the only score able to predict mortality significantly better than the reference line, with an AUC (95%CI) of 0.71 (95%CI: 0.54-0.88, $P = 0.021$). Among patients with AD, all prognostic scores performed significantly better than the reference line regarding 28-d mortality, presenting with similar AUCs: CLIF-C AD score 0.75 (95%CI: 0.63-0.88), CP score 0.72 (95%CI: 0.59-0.85), MELD score 0.75 (95%CI: 0.61-0.90), MELD-Na score 0.76 (95%CI: 0.61-0.90), and CLIF-C OF score 0.74 (95%CI: 0.60-0.88). The same occurred concerning AUCs for 90-d mortality: CLIF-C AD score 0.70 (95%CI: 0.57-0.82), CP score 0.73 (95%CI: 0.62-0.84), MELD score 0.71 (95%CI: 0.59-0.83), MELD-Na score 0.73 (95%CI: 0.62-0.84), and CLIF-C OF score 0.65 (95%CI: 0.52-0.78).

CONCLUSION

This study demonstrated that CLIF-C ACLF is the best available score for the prediction of 28-d mortality among patients with ACLF. CLIF-C AD score is also useful for the prediction of mortality among cirrhotic patients with AD not fulfilling diagnostic criteria for ACLF, but it was not superior to other well-established prognostic scores.

Key words: Cirrhosis; Acute-on-chronic liver failure; Mortality; Prediction; Prognosis; Acute decompensation of cirrhosis

Core tip: The present study demonstrated that chronic liver failure-consortium (CLIF-C) acute-on-chronic liver failure score (ACLF) is the best available score for the prediction of 28-d mortality among patients with ACLF, but it was unable to determine the same regarding 90-d mortality. On the other hand, while this study also demonstrated that CLIF-C acute decompensation (AD) was useful for the prediction of 28-d and 90-d mortalities among patients with AD not fulfilling diagnostic criteria for ACLF, it failed to identify superiority when compared to other scores already routinely used worldwide.

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INTRODUCTION

Cirrhosis is a relevant disease worldwide, both because of its high prevalence and because of its high mortality. In addition, this disease is responsible for substantial use of health care resources[1]. In Brazil, for instance, liver diseases are the eighth leading cause of death, and cirrhosis is the major cause of hospital admissions and lethality among them. Moreover, the burden of cirrhosis in terms of hospital admissions and mortality rate is still increasing, despite considerable medical advances[2]. Most of cirrhosis-related admissions and deaths are related to its acute decompensations.

Recently, a redefinition of acute-on-chronic liver failure (ACLF), an especially severe form of cirrhosis decompensation based on the occurrence of organic dysfunctions, was proposed by the European Association for the Study of the Liver - Chronic liver failure (CLIF) consortium. ACLF represents a syndrome occurring in patients with chronic liver disease, which is characterized by acute deterioration of liver function and one or more extrahepatic organ failures, leading to increased short-term mortality[3]. In the United States, from 2001 to 2011, the proportion of patients discharged from hospitals with a diagnosis of cirrhosis increased from 0.4% to 4.0%, while the proportion of those discharged with a diagnosis of ACLF increased from 0.3% to 6.0%[1]. Similarly, a recent French study demonstrated that, between 2008 and 2013, the annual proportion of cirrhotic patients with ACLF undergoing orthotopic liver transplantation (OLT) increased from 32% to 51%[4].

Considering the need to better predict the prognosis of patients with acute decompensations of cirrhosis, the CLIF Consortium proposed the use of two scores: one for patients with ACLF and the other for patients with acute decompensation (AD), but not ACLF[5,6]. Considering the lack of evidence about the performance of these scores outside Europe and taking into account possible differences regarding the epidemiological aspects of liver diseases and the characteristics of health care systems, this study aimed at validating their use in a Brazilian population.

MATERIALS AND METHODS

Study design and setting

This was a prospective cohort study conducted with a convenience consecutive sample of patients admitted

to a tertiary hospital of Porto Alegre, southern Brazil. All participants were adults admitted to the Emergency Department of Nossa Senhora da Conceição Hospital with either acute decompensation of cirrhosis - AD group - or acute-on-chronic liver failure - ACLF group[3]. The AD group was further subdivided in two: (1) patients with AD that did not develop ACLF during hospital stay - hereafter AD group; and (2) patients admitted with AD that did develop ACLF at some point during the same hospitalization - henceforth AD evolving to ACLF group.

Outcome

The evaluated outcome was transplant-free survival according to the diagnosis of AD or ACLF.

Predictors

In order to evaluate the capacity of Chronic Liver Failure-Consortium Acute Decompensation (CLIF-C AD)[6] and Chronic Liver Failure-Consortium Acute-on-Chronic Liver Failure (CLIF-C ACLF) scores[5] in predicting death in 28 d and 90 d after the diagnosis, their sensitivity, specificity and positive and negative predictive values were calculated, and receiver operating characteristic (ROC) curves were developed. Several well-established severity scores employed for cirrhosis assessment were used as comparators: Model for End-Stage Liver Disease (MELD), MELD Sodium (MELD-Na), Child-Pugh (CP) score, and the Chronic Liver Failure-Consortium Organ Failure (CLIF-C OF) score[5-9].

Participants

Between January and September 2016, patients over 18 years-old admitted to the Emergency Department were screened by the International Classification of Diseases - 10th revision (ICD-10) codes and deemed eligible if codes K70 to K77 were stated at their hospital admission forms. Participants were cirrhotic patients undergoing a non-elective Emergency Department admission for acute decompensation - *i.e.,* ascites, encephalopathy, gastrointestinal hemorrhage, or bacterial infection according to criteria stated by Moreau *et al*[3]. Cirrhosis was diagnosed based on histology or on clinical grounds, laboratory tests, imaging and endoscopic findings. Exclusion criteria were (1) elective hospitalization; and (2) non-elective Emergency Department admission for reasons different from acute decompensation of cirrhosis. For patients undergoing more than one hospitalization during the studied period, only data regarding the first admission was considered for analysis. Participants were followed until the end of December 2016.

Data collection

Data were drawn from the electronic medical records of the patients. Data collection did not affect management of participants during hospital stay. Data extraction was carried-out on a pilot-tested Microsoft Excel™ spreadsheet by two authors (FSB and RVP).

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional Ethical Committee. Informed consent was waived by the Ethical Committee.

Statistical analysis

Qualitative data were described as proportions, and Pearson's χ^2 or Fisher's exact test was used for inferential analysis. Quantitative data were described as means ± SD, and analysis of variance (ANOVA) was used for comparison across three groups. Alpha was set at 0.05 and all comparisons were two-tailed.

OLT was regarded as a competing end-point, thus Kaplan-Meier survival analysis was undertaken assessing time to event as days from AD or ACLF diagnosis to death or OLT. Individuals who did not die nor were submitted to OLT were censored at the end of follow-up. Mantel-Cox log rank statistic was employed for inferential analysis across groups. Median survival times and their respective 95%CI according to group were obtained.

ROC curves, their corresponding areas under the curve (AUC) and respective AUC 95%CI were used to assess the performance of the CLIF-C AD score, CLIF-C ACLF score, CLIF-C OF score, MELD, MELD-Na, and CP score at 28 and 90 d from diagnosis. Patients lost to follow-up or with incomplete data were excluded from such analyses.

A univariate analysis was performed using all the scores and selected baseline clinical characteristics not embedded in the prediction scores as covariates and death at any point during the study as the dependent variable. Data analysis was performed using the Statistical Package for Social Sciences™ version 18.0.

RESULTS

One hundred seventeen patients were considered eligible for the study, and 113 individuals fulfilled all the inclusion criteria. At admission, 18 (15.9%) patients had ACLF and 95 (84.1%) individuals had AD without ACLF, of which 24 eventually develop ACLF during the course of hospitalization (AD evolving to ACLF group). Figure 1 shows the flowchart of patients through the study.

Tables 1 and 2 describe, respectively, baseline clinical characteristics and laboratory findings of patients according to group. Of note, none of the patients from the AD group was admitted for spontaneous bacterial peritonitis (SBP), while 3 (12.5%) patients from the AD evolving to ACLF group and 2 (11.1%) of those from the ACLF group were admitted for SBP (P = 0.035). Over 70.0% of patients who were diagnosed with ACLF had ACLF grade 1. Kidney failure was the single most frequent organic failure, being present in 66.7% of patients with ACLF at admission and in 75.0% of

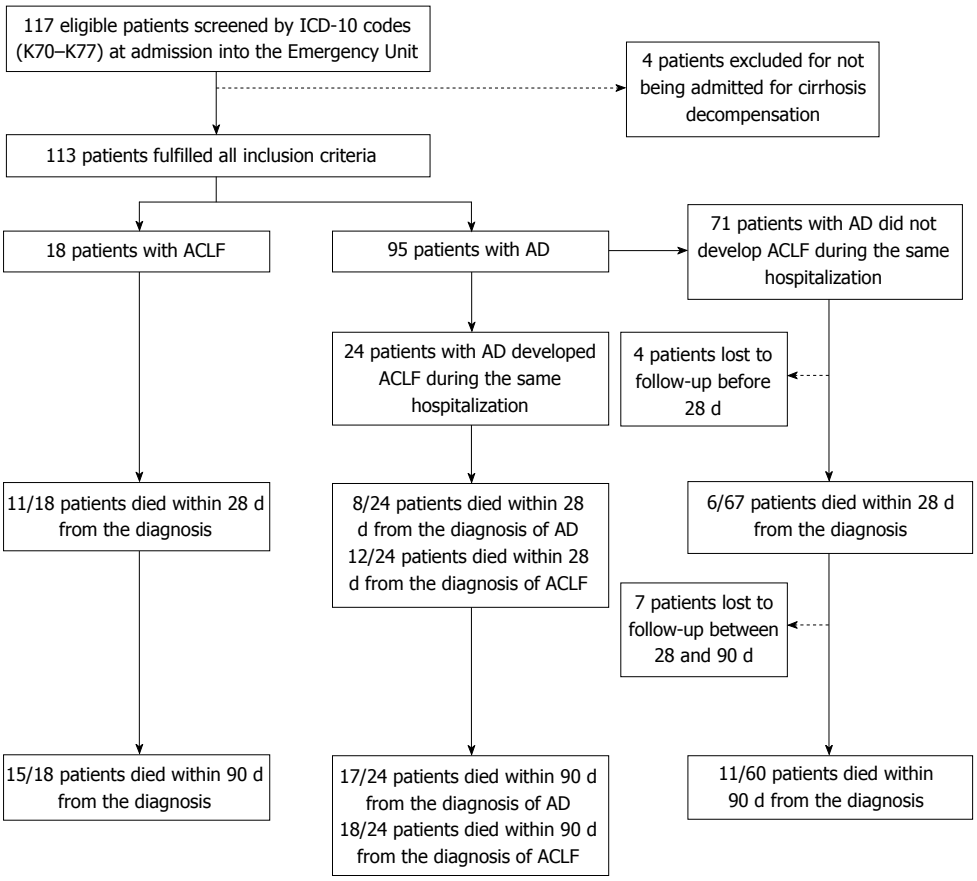

Figure 1 Flowchart of patients through the study. No patient was submitted to orthotopic liver transplantation. ICD-10: International Classification of Diseases, 10th revision; ACLF: Acute-on-chronic liver failure; AD: Acute decompensation of cirrhosis.

those with AD evolving to ACLF. Moreover, mean serum C-reactive protein was approximately three times higher in the ACLF group compared to the AD group (77.5 mg/dL *vs* 25.5 mg/dL, $P = 0.003$).

Median survival times for ACLF group (11.0 d) and AD evolving to ACLF group (32.0 d) did not differ significantly ($P = 0.247$). Merging the ACLF and the AD evolving to ACLF groups into a single cohort yielded a median survival time of 27.0 d (95%CI: 7.97-46.03; data not shown), which differed significantly from the median survival of 239 d of the AD group ($P < 0.001$). The AD group had significantly lower 28-d (9.0%) and 90-d (18.3%) mortality as compared to the AD evolving to ACLF group and to the ACLF group (both $P < 0.001$). The latter groups had comparable 28-d and 90-d mortalities ($P = 0.542$ and $P = 0.708$, respectively). At 28 d from the diagnosis of ACLF (either at admission or during hospitalization), 54.8% (23/42) of patients had died, whereas, at 90 d, 78.6% (33/42) of them had died. One patient from the ACLF group and seven others from the AD group died after 90 d of follow-up. Kaplan-Meier analysis revealed that patients with ACLF grades 1, 2, and 3 had significantly different survivals (Mantel-Cox $P = 0.031$), with median survival times of 29.0 d (95%CI: 4.3-53.7), 15.0 d (95%CI: 0.0-52.2), and 3.7 d (95%CI: 0.0-14.2) respectively (data not shown). No patient was submitted to OLT. Figure 2 depicts the Kaplan-Meier survival analysis, and Table 3 shows median survival times and mortalities according to group.

Figure 3 illustrates the ROC curves for mortality of several prognostic scores at 28 and 90 d from the diagnosis of either AD or ACLF. Since 24 patients developed ACLF during hospitalization, they contributed with data to both the CLIF-C AD and the CLIF-C ACLF ROC curves. One patient from the AD group did not have sufficient data to calculate CLIF-C AD score and was excluded from ROC curve analyses. Four patients from the AD group had follow-ups shorter than 28 d, hence they were also excluded from the 28-d and 90-d ROC curves. Furthermore, another seven individuals from the AD group were lost to follow-up between 28 and 90 d and were excluded from the 90-d ROC curve. Among patients with ACLF, at 28 d from the diagnosis, CLIF-C ACLF was the only score able to predict mortality significantly better than the reference line, with an AUC of 0.71 (95%CI: 0.54-0.88, $P = 0.021$). Considering a pretest probability of death at 28 d of 54.8% (23/42 patients with the diagnosis of ACLF at some point of the study), a CLIF-C ACLF score cut-off of 40 points yielded 100% sensitivity (Se), 37% specificity (Sp), 100% negative predictive value (NPV), and 65.8% positive predictive value (PPV), whereas a cut-off of 60 points yielded 4% Se, 100% Sp, 46.2%

Table 1 Baseline clinical characteristics and acute-on-chronic liver failure grade according to group

	AD (n = 71)		AD evolving to ACLF (n = 24)		ACLF (n = 18)		ANOVA/ pearson χ^2
	No. mean	% SD	No. mean	% SD	No. mean	% SD	*P* value
Sex							0.259
Male	48	67.6%	13	54.2%	14	77.8%	
Age (yr)	57.8	10.2	62.6	8.0	61.3	12.9	0.097
Primary etiology of cirrhosis							0.592
Alcohol	23	32.4%	11	45.8%	9	50.0%	
HBV	4	5.6%	0	0.0%	0	0.0%	
HCV	34	47.9%	9	37.5%	6	33.3%	
NASH	4	5.6%	1	4.2%	2	11.1%	
Other	6	8.5%	3	12.5%	1	5.6%	
Main cause of current hospital admission							0.035
SBP	0	0.0%	3	12.5%	2	11.1%	
Ascites - not SBP	20	28.2%	7	29.2%	1	5.6%	
Encephalopathy	15	21.1%	6	25.0%	5	27.8%	
Alcoholic hepatitis	2	2.8%	2	8.3%	1	5.6%	
Digestive bleeding	21	29.6%	2	8.3%	4	22.2%	
Sepsis	0	0.0%	0	0.0%	1	5.6%	
Other	13	18.3%	4	16.7%	4	22.2%	
No. of previous hospitalizations	2.4	4.7	2.5	3.4	2.2	3	0.972
Alcohol consumption in the past three months							0.683
Yes	10	14.1%	6	25.0%	3	16.7%	
West Haven HE grade at admission							0.005
No HE	54	76.1%	15	62.5%	7	38.9%	
Grades Ⅰ and Ⅱ	13	18.3%	9	37.5%	7	38.9%	
Grades Ⅲ and Ⅳ	4	5.6%	0	0.0%	4	22.2%	
Vasopressor at admission							0.070
Yes	0	0.0%	0	0.0%	1	5.6%	
Mechanical ventilation at admission							< 0.001
Yes	0	0.0%	0	0.0%	3	16.7%	
Mean arterial pressure (mmHg), n = 111	95.5	18.3	95.7	18.0	79.9	22.7	0.008
PaO_2/FiO_2 (mmHg), n = 17	476.0	173.5	380.0	No SD (n = 1)	352.4	152.5	0.341
SpO_2/FiO_2, n = 113	461.4	21.9	457.3	16.6	399.7	130.7	< 0.001
Child-Pugh score, n = 110	8.0	2.0	9.9	1.9	10.3	1.8	< 0.001
MELD score, n = 111	13.0	3.6	21.7	6.5	23.4	7.2	< 0.001
MELD-Na score, n = 111	14.0	4.6	23.8	5.8	23.9	8.9	< 0.001
CLIF-C OF score, n = 111	6.5	0.8	6.9	1.0	9.1	1.7	< 0.001
ACLF grade[1]							
ACLF grade 1	NA	NA	17	70.8%	13	72.2%	1.000
ACLF grade 2	NA	NA	2	8.3%	4	22.2%	0.375[2]
ACLF grade 3	NA	NA	5	20.8%	1	5.6%	0.214[2]

[1]For AD evolving to ACLF group, ACLF grade refers to classification at the time of ACLF diagnosis, not at the time of hospital admission; [2]Fisher's exact test. AD: Acute decompensation of cirrhosis; ACLF: Acute-on-chronic liver failure; ANOVA: Analysis of variance; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NASH: Non-alcoholic steatohepatitis; SBP: Spontaneous bacterial peritonitis; HE: Hepatic encephalopathy; PaO_2: Arterial oxygen pressure; FiO_2: Inhaled oxygen fraction; SpO_2: Partial oxygen saturation; MELD: Model for End-Stage Liver Disease; MELD-Na: MELD sodium; CLIF-C OF: Chronic Liver Failure Consortium Organ Failure; NA: Not applicable.

NPV, and 100% PPV. The optimum cut-off value for the present study was 43.9 points, which returned 82.6% Se and 57.9% Sp. None of the several prognostic scores was superior to the reference line regarding 90-d mortality.

Regarding 28-d mortality among patients with AD, all the prognostic scores performed significantly better than the reference line and had similar AUCs when compared to each other: CLIF-C AD score 0.75 (95%CI: 0.63-0.88), CP score 0.72 (95%CI: 0.59-0.85), MELD score 0.75 (95%CI: 0.61-0.90), MELD-Na score 0.76 (95%CI: 0.61-0.90), and CLIF-C OF score 0.74 (95%CI: 0.60-0.88). At 90 d from the diagnosis, all prognostic scores also performed significantly better than the reference line, with comparable AUCs: CLIF-C AD score 0.70 (95%CI: 0.57-0.82), CP score 0.73 (95%CI: 0.62-0.84), MELD score 0.71 (95%CI: 0.59-0.83), MELD-Na score 0.73 (95%CI: 0.62-0.84), and CLIF-C OF score 0.65 (95%CI: 0.52-0.78). Considering a pretest probability of death at 90 d of 33.3% (28/84 patients; 95 patients with AD at admission minus 11 individuals lost to follow-up), a CLIF-C AD score cut-off of 45 points yielded 93% Se, 20% Sp, 85.1% NPV, and 36.7% PPV, while a cut-off of 60 points yielded 25% Se, 90.9% Sp, 70.8% NPV, and 57.8% PPV. The optimum cut-off value for this study was 53.4 points, which returned 65.5% Se and 72.2% Sp.

Univariate analysis for death at any given time

Table 2 Baseline laboratory findings according to group

	AD (n = 71)		AD evolving to ACLF (n = 24)		ACLF (n = 18)		ANOVA
	Mean	SD	Mean	SD	Mean	SD	*P* value
Total bilirubin (mg/dL), n = 112	2.1	2.2	5.1	5.3	5.7	7.2	< 0.001
Serum creatinine (mg/dL), n = 113	1.0	0.3	1.2	0.4	2.1	0.8	< 0.001
INR, n = 112	1.4	0.3	1.5	0.5	1.8	0.4	< 0.001
White-cell count (109 cells/L), n = 113	6.9	3.7	8.8	3.6	12.6	6.1	< 0.001
Serum sodium (mmol/L), n = 113	138.4	3.9	136.1	4.3	137.6	5.6	0.070
Serum albumin (g/dL), n = 112	3.0	0.6	2.7	0.5	2.7	0.5	0.006
Serum C-reactive protein (mg/L), n = 47	25.5	33.0	41.9	32.0	77.5	61.7	0.003

AD: Acute decompensation of cirrhosis; ACLF: Acute-on-chronic liver failure; ANOVA: Analysis of variance; INR: International normalized ratio.

Table 3 Survival time and mortality according to group

Group (group symbol)	Survival time[1]			Mortality[1]		
	Median (d)		Mantel-Cox	28-d mortality	90-d mortality	Death at any point during the study
	Estimate	(95%CI)	*P* value	n (%)	n (%)	n (%)
AD (A), n = 71	239	(166.4-311.6)	Reference group	6/67 (9.0)[2]	11/60 (18.3)[2]	18/60 (30.1)[2]
AD evolving to ACLF (B), n = 24	32	(0.0-64.4)	B *vs* A < 0.001; B *vs* C = 0.247	12/24 (50.0)[2,3]	18/24 (75.0)[2,3]	18/24 (75.0)[2,3]
ACLF (C), n = 18	11	(2.7-19.3)	C *vs* A < 0.001; B + C *vs* A < 0.001	11/18 (61.1)[2,3]	15/18 (83.3)[2,3]	16/18 (88.9)[2,3]
Overall, n = 113	165	(117.7-212.3)	Not applicable	29/109 (26.6)	44/102 (43.1)	52/102 (51.0)

[1]Survival time and mortality for group B regards to time elapsed from the diagnosis of ACLF; [2]Pearson's χ^2 *P* values < 0.001 for comparisons across all groups; [3]Fisher's exact test *P* values for comparisons between groups B and C were 0.542, 0.708, and 0.431 for 28-d mortality, 90-d mortality, and death at any point during the study, respectively. AD: Acute decompensation of cirrhosis; ACLF: Acute-on-chronic liver failure.

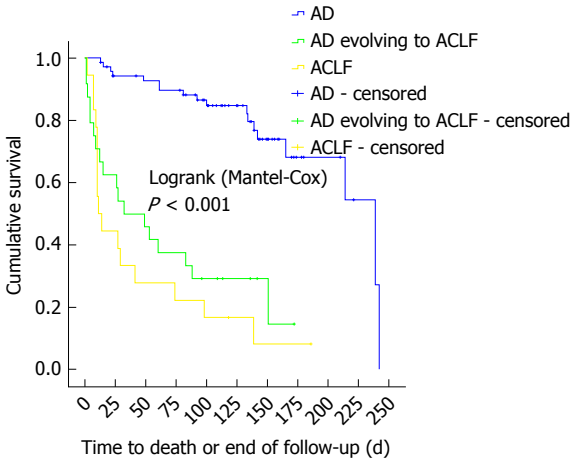

Figure 2 Kaplan-Meier survival analysis. AD: Acute decompensation; ACLF: Acute-on-chronic liver failure.

during the study was performed. The covariates evaluated were sex, alcohol ingestion three months prior to admission, alcohol-induced cirrhosis, digestive bleeding, SBP, history of any previous hospitalization, CLIF-C OF score, CLIF-C AD score, CLIF-C ACLF score, CP score, MELD, and MELD-Na. According to the univariate analysis, the only variable associated to death was CLIF-C ACLF score (P = 0.009). Considering that the univariate analysis identified only one variable associated to this outcome, a multivariate analysis was not performed.

DISCUSSION

Considering the high prevalence, mortality and impact on the healthcare systems associated to cirrhosis decompensations, it is of great importance to develop tools which could better predict outcomes of cirrhotic patients[1]. The present study demonstrated that CLIF-C ACLF is the best available score for the prediction of 28-d mortality among patients with ACLF, as previously suggested[5], but it was unable to demonstrate the same regarding 90-d mortality, probably because most of the deaths in this group of patients occurred early in their follow-up. On the other hand, while this study also demonstrated that CLIF-C AD was useful for the prediction of 28-d and 90-d mortalities among patients with AD not fulfilling diagnostic criteria for ACLF, it failed to identify superiority when compared to other scores, some of them already routinely used worldwide. This finding differs from that of the study that originally proposed CLIF-C AD score, in which it performed better than other scores, at least for the prediction of 90-d mortality[6]. This could be explained by our sample of patients with AD not being large enough in order to detect a difference between the performance of CLIF-C AD score and that of other prognostic scores. Nevertheless, it is important to remind that our sample was sufficiently large to demonstrate that CLIF-C AD score had an AUC significantly greater than that of the

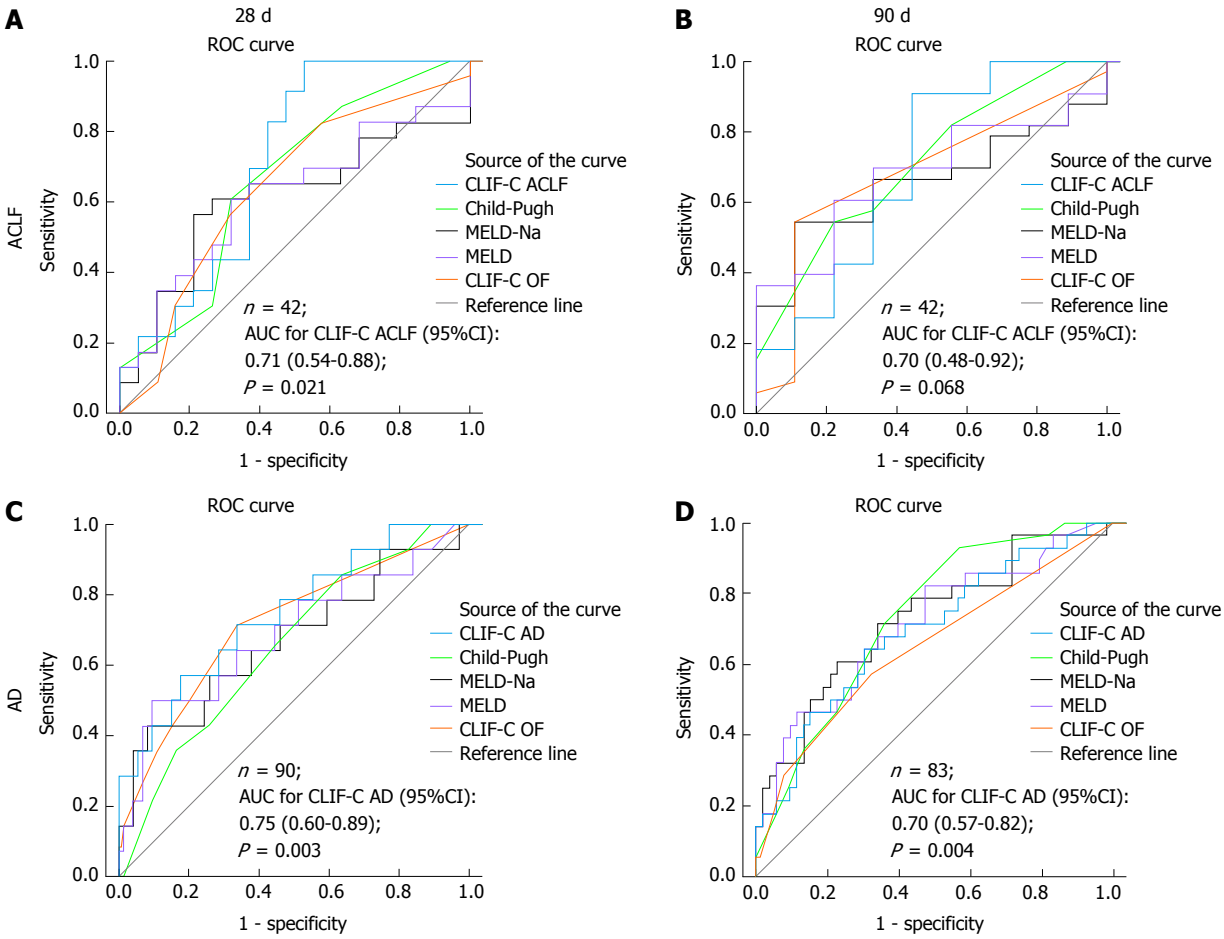

Figure 3 Receiver operating characteristic curves of prognostic scores regarding 28-d and 90-d mortality. A: Receiver operating characteristic (ROC) curves of several scores regarding 28-d mortality in patients diagnosed with acute-on-chronic liver failure (ACLF). B: ROC curves of several scores regarding 90-d mortality in patients diagnosed with ACLF. C: ROC curves of several scores regarding 28-d mortality in patients diagnosed with acute decompensation of cirrhosis (AD). D: ROC curves of several scores regarding 90-d mortality in patients diagnosed with AD. CLIF-C ACLF: Chronic liver failure-consortium acute-on-chronic liver failure score; CLIF-C AD: Chronic liver failure-consortium acute decompensation score; MELD: Model for End-Stage Liver Disease; MELD-Na: MELD sodium; CLIF-C OF: Chronic liver failure-consortium organ failure score; AUC: Area under the curve.

reference line.

In the present study, ACLF was diagnosed in 37% of patients with decompensated cirrhosis, which is similar to what was previously reported[10]. The group of patients that fulfilled the diagnostic criteria for ACLF had a median survival time of 27 d, which differed significantly from the median survival of 239 d of the AD group ($P < 0.001$), corroborating the idea that ACLF is a distinct syndrome with an elevated short-term mortality[3]. Moreover, as also previously described[3], the prognosis of patients diagnosed with ACLF at hospital admission was not significantly different from that of patients who developed ACLF during hospitalization. Therefore, patients should be managed similarly irrespective of the moment of the diagnosis of ACLF.

The poor prognosis of ACLF was also demonstrated in a North American population of infected cirrhotic patients. In that study, 30-d survival was 51.3% for patients with ACLF and 91.4% for those without ACLF[11], which is similar to what we found (28-d survival of 38.9% for patients with ACLF and 91% for those without ACLF). Nevertheless, it is important to highlight that the definition of ACLF used in that study is not the same as that suggested by the CLIF Consortium and used in the present study.

Using the CLIF Consortium definition of ACLF, Soares e Silva *et al*[12] also showed that ACLF was a strong predictor of short-term mortality in a Brazilian population. Similar findings were described as well in a recent Argentina study[13]. However, to the best of our knowledge, the present study is the first to validate CLIF-C AD and CLIF-C ACLF scores outside Europe.

Other interesting finding of our study is that patients with ACLF more frequently had alcohol-related cirrhosis and less frequently had hepatitis C virus. These results are similar to those reported in the CANONIC study[3] and also corroborate what Jeong *et al*[14] have shown in their paper.

ACLF differs from traditional decompensated cirrhosis, not only because of the presence of organ failure(s) and higher mortality rate, but also because it happens more frequently in younger patients and in those with an alcoholic etiology of cirrhosis, as

well as because it is associated to higher prevalences of some precipitating events (bacterial infections, active alcoholism, among others), and to a higher level of systemic inflammation[10]. As expected, in our study, we found that serum C-reactive protein and leukocyte count were significantly higher in the ACLF group compared to the AD group. This corroborates the systemic inflammation hypothesis[15], which suggests that ACLF is caused by an aggravation of systemic inflammation and by the associated systemic circulatory dysfunction already present in decompensated cirrhosis. According to this hypothesis, these processes would lead to organ failures as a consequence of hypoperfusion and the direct deleterious effects of inflammatory mediators on microcirculation and on cell physiology homeostasis[15]. The systemic inflammation hypothesis was based on the CANONIC study, in which it was observed a close relationship between blood leukocytes and C-reactive protein levels and the presence and severity of ACLF[3]. In this context, the association between the course of systemic inflammation and the course of ACLF was also recently demonstrated[16]. The relevance of the inflammatory state to the prognosis of cirrhotic patients is such that prognostic models incorporating C-reactive protein are also being studied[17,18].

Among the strengths of the present study, one must highlight its prospective character and the large sample of patients enrolled in a single centre. On the other hand, this study also presents limitations. One of the limitations, contradictorily, regards the fact that this was a single-center study, and its results might not reflect the reality elsewhere. For instance, despite being one of the largest hospitals in southern Brazil, our institution does not have a liver transplantation program, which does not preclude patients from being referred to others hospitals, but might explain why none of the studied patients was submitted to OLT. Nevertheless, we understand that the characteristics of our institution are similar to those of the majority of tertiary hospitals in Brazil and probably in Latin America and, therefore, we believe in the external validity of our findings.

Another limitation is the fact that we did not perform a sequential assessment of ACLF status and of the scores of the patients during hospitalization, which has recently been demonstrated useful[19,20]. This assessment was not planned prior to data collection, and we did not have sufficient data to perform it.

Yet another limitation of this study concerns missing data for some of the evaluated parameters, which is explained by the fact that authors did not interfere with the management of the patients. However, it should be noticed that the main analyses of this study were not affected by missing data.

In conclusion, in order to improve the quality of care of cirrhotic patients, it is of the utmost importance to be able to accurately predict the prognosis of decompensated cirrhosis. This study has shown that CLIF-C ACLF is the most accurate score to predict mortality of patients with ACLF in a Brazilian setting. Moreover, it has demonstrated that CLIF-C AD score is also useful for the prediction of mortality among cirrhotic patients with AD not fulfilling diagnostic criteria for ACLF, but it was not superior to other well-established prognostic scores.

COMMENTS

Background

Most of cirrhosis-related admissions and deaths are related to acute decompensations.

Research frontiers

Acute-on-chronic liver failure (ACLF) has a significantly higher mortality compared to other forms of acute decompensation (AD) of cirrhosis. Two recently developed scores - CLIF-C ACLF and CLIF-C AD - were proposed as tools for assessing prognosis of ACLF and AD. They were validated in European patients.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to validate CLIF-C AD and CLIF-C ACLF scores outside Europe.

Applications

In order to aid clinical decision-making and to improve the quality of care of cirrhotic patients, it is of the utmost importance to accurately predict the prognosis of AD and ACLF. This study has shown that CLIF-C ACLF is the most accurate score to predict mortality of patients with ACLF in a Brazilian setting. Moreover, it has also shown that CLIF-C AD score is also useful to predict mortality among cirrhotic patients with AD not fulfilling diagnostic criteria for ACLF, but it was not superior to other well-established prognostic scores.

Terminology

ACLF: acute-on-chronic liver failure - *i.e.*, acute decompensation of cirrhosis with at leats two organ failures, or one organ failure plus renal dysfunction, or one organ failure plus mild encephalopathy, or isolated renal failure; AD: acute decompensation of cirrhosis - *i.e.*, digestive bleeding, large-volume ascites, hepatic encephalopathy, or bacterial infection.

Peer-review

The manuscript is novel one describing one of the most important issues in the diagnosis and mangement of decompensated and liver failure cases but some comments are to be considered: As the definition of ACLF it is a specific syndrome characterized by AD, organ failure and high short-term mortality.

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CASE REPORT

Refractory hepatic encephalopathy in a patient with hypothyroidism: Another element in ammonia metabolism

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Abstract

Hepatic encephalopathy (HE) remains a diagnosis of exclusion due to the lack of specific signs and symptoms. Refractory HE is an uncommon but serious condition that requires the search of hidden precipitating events (*i.e.*, portosystemic shunt) and alternative

diagnosis. Hypothyroidism shares clinical manifestations with HE and is usually considered within the differential diagnosis of HE. Here, we describe a patient with refractory HE who presented a large portosystemic shunt and post-ablative hypothyroidism. Her cognitive impairment, hyperammonaemia, electroencephalograph alterations, impaired neuropsychological performance, and magnetic resonance imaging and spectroscopy disturbances were highly suggestive of HE, paralleled the course of hypothyroidism and normalized after thyroid hormone replacement. There was no need for intervention over the portosystemic shunt. The case findings support that hypothyroidism may precipitate HE in cirrhotic patients by inducing hyperammonaemia and/or enhancing ammonia brain toxicity. This case led us to consider hypothyroidism not only in the differential diagnosis but also as a precipitating factor of HE.

Key words: Hepatic encephalopathy; Hypothyroidism; Cognitive impairment; Cirrhosis; Portosystemic shunt; Magnetic resonance spectroscopy

Core tip: Hepatic encephalopathy (HE) remains a diagnosis of exclusion due to the lack of specific signs and symptoms. Refractory HE requires the search of hidden precipitating events and alternative diagnosis. We describe a patient with refractory HE who presented with large portosystemic shunt and post-ablative hypothyroidism. Her cognitive impairment, hyperammonaemia, electroencephalograph alterations, impaired neuropsychological performance and magnetic resonance imaging and spectroscopy disturbances suggestive of HE paralleled the course of hypothyroidism and improved after thyroid hormone replacement. The case findings support that hypothyroidism may precipitate HE in cirrhotic patients by inducing hyperammonaemia and/or enhancing ammonia brain toxicity.

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INTRODUCTION

Hepatic encephalopathy (HE) is a brain dysfunction that frequently affects patients with chronic liver failure. Its symptoms and signs are not specific and may also be present in other causes of brain dysfunction[1]. In cirrhotic patients, an episode of HE is usually triggered by a clinical event that exacerbates the toxic effects of ammonia and other substances on the brain. The search and adequate treatment of such precipitating factors are fundamental to revert the HE of these patients. Refractory HE is an uncommon but serious condition that requires the search of alternative diagnosis and non-identified precipitating factors.

Hypothyroidism shares clinical manifestations with HE, such as disturbances in memory and attention, and is considered in the differential diagnosis of HE. However, little is known on whether hypothyroidism coexists with or precipitates HE. Herein, we describe a patient with a large portosystemic shunt who developed refractory HE during post-ablative hypothyroidism. The case's clinical manifestations, electroencephalographic pattern and magnetic resonance imaging (MRI) and spectroscopy (MRS) findings were consistent with HE. Treatment with thyroid hormone normalized ammonia levels and improved clinical alterations and electroencephalograph (EEG). MRI and MRS showed an improvement in markers of ammonia toxicity, suggesting that hypothyroidism might increase brain exposition to ammonia and precipitate HE. This case led us to consider hypothyroidism not only in the differential diagnosis, but also as a precipitating factor of HE.

CASE REPORT

A 58-year-old woman with the diagnosis of chronic and refractory HE in the setting of liver cirrhosis and large portosystemic shunt was referred to our Liver Unit from a secondary hospital. Her liver disease was diagnosed in 2011 when she presented her first episode of overt HE (type C, grade Ⅱ). Due to a history of alcohol abuse for years and other causes of liver disease being ruled out, a diagnosis of alcoholic cirrhosis Child-Pugh B7 was established. The patient had no ascites nor hepatocellular carcinoma, and primary prophylaxis with propranolol because of oesophageal varices grade Ⅱ-Ⅲ/Ⅳ was started. At that time, the patient abandoned alcohol consumption, and remained asymptomatic and compensated from the liver point of view.

As comorbidities, the patient had diabetes mellitus, arterial hypertension and multinodular goitre diagnosed in 2011 in a pre-toxic state. In January 2012, she started carbimazole and received radioiodine therapy in February 2012 because of hyperthyroidism. Carbimazole was stopped after 4 mo, and treatment with levothyroxine (October 2012-April 2013) was subsequently started because of post-ablative hypothyroidism. In August 2013, carbimazole was restarted due to recurrent toxic multinodular goitre, and a second session of radioiodine was administered in November 2014. Carbimazole was stopped at the end of November 2014 and levothyroxine was again prescribed in middle of January 2015 because of

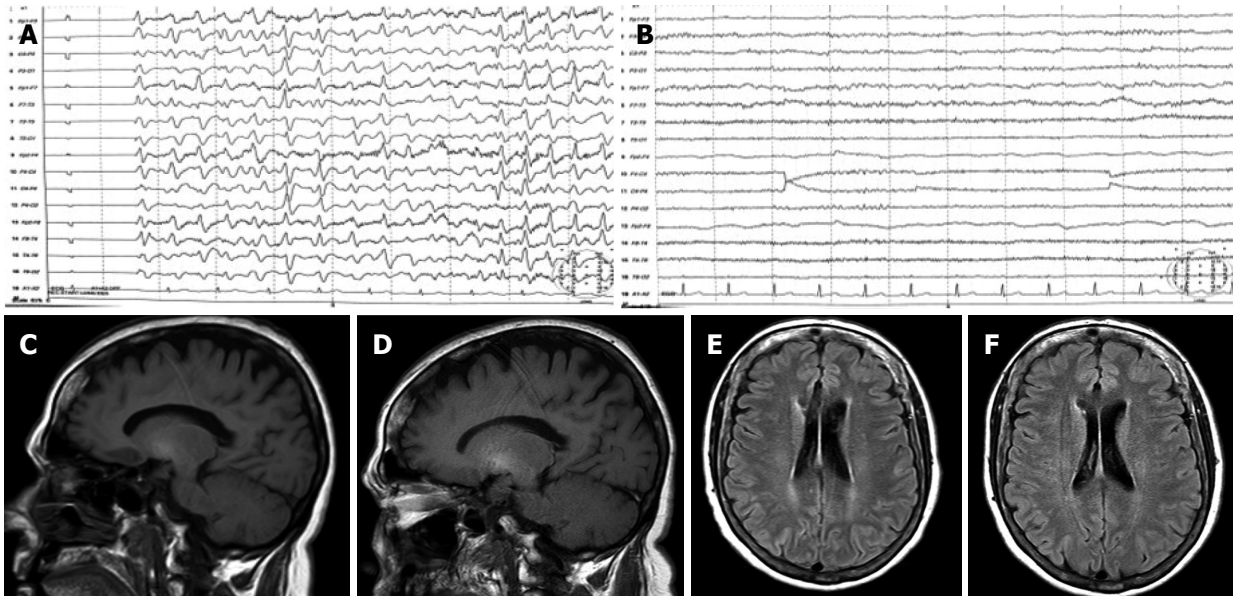

Figure 1 Electroencephalograph and magnetic resonance findings at baseline and after treatment with thyroid hormone. A: EEG at baseline showing slow activity and generalized triphasic waves; B: EEG after treatment showing normalization of brain activity; C: T1-weighted MRI showed high-signal in basal ganglia at baseline; D: T1-weighted MRI performed after thyroid hormone replacement showed non-significant changes; E: FLAIR MRI sequence at baseline demonstrated subtle periventricular white matter hyperintensities; F: FLAIR MRI image after thyroid hormone replacement demonstrated a normalization of periventricular white matter signal abnormalities. EEG: Electroencephalograph; MRI: Magnetic resonance imaging.

subclinical hypothyroidism (thyroid-stimulating hormone (TSH) 8 mUI/L, normal range: 0.5-4.5 mUI/L).

In February 2015, the patient was admitted to another hospital because of a subacute neurological syndrome consistent with daily hypersomnia, dysarthria, mental slowness, bizarre behaviour ("she wanted to turn on the TV with her keys") and diminished voluntary movements. At the physical exam, she was conscious and oriented, although she presented mental slowness and slow speech rate, shortened attention span and irritability. The patient had asterixis, with no other evident motor or neurological alterations. Laboratory test showed mild macrocytosis, with no other abnormalities and the brain computed tomography (CT) scan was also normal. Precipitating factors were not found (no infection, gastrointestinal bleeding or recent alcohol abuse was documented) and oral and rectal lactulose was started, producing adequate laxative response. However, rifaximin was added because of lack of clinical efficacy.

An EEG was performed and showed slow activity with the presence of generalized triphasic waves (Figure 1A). Because of persistent neurological impairment consistent with HE, an abdominal CT scan searching for portosystemic shunts was performed and revealed a large shunt communicating portal and left renal veins. The patient was then referred to our tertiary hospital for shunt embolization.

At admission, the patient showed similar neurological status with hypersomnia, mental slowness, shortened attention span, and memory difficulties, but was oriented in time and space. Asterixis and temporal disorientation was occasionally present but other neurological abnormalities were not observed. She reported pronounced constipation and gained weight of 3 kg within the last weeks. During her stay in the hospital, she remained neurologically stable despite high doses of oral lactulose (with adequate daily bowel movements) and rifaximin, developing occasionally confusion, disorientation and asterixis without clear precipitating factor and with partial response to lactulose intensification.

Laboratory testing (Table 1) showed macrocytosis without anaemia and hyperammonaemia and thyroid test demonstrated hypothyroidism (TSH 70 mUI/L and T4 0.6 ng/dL; normal range: 0.6-1.4 ng/dL). A Psychometric Hepatic Encephalopathy Score (PHES) was performed and showed a pathological performance with a score of 9 SD below the reference population (Table 1). MRI and MRS were performed using 1.5T equipment (Figure 1C-F). T1-weighted hyperintensity in basal ganglia (Figure 1C) and periventricular white matter high signals on FLAIR sequence were shown (Figure 1E).

A therapeutic decision of treating and resolving first hypothyroidism and re-evaluating the patient afterwards was based on three aspects: (1) time course (neurological impairment is coincident with the progression of thyroid disturbance); (2) lack of previous neurological impairment attributable to portosystemic shunt (completely asymptomatic over the past 4 year); and (3) although many symptoms of the patient could be attributable to either hypothyroidism or HE, some others (constipation and weight gain) were highly indicative of thyroid dysfunction that could potentially contribute to the clinical syndrome.

Table 1 Anthropometric, laboratory, neuropsychological and spectroscopic data of the patient at hospital admission and 1 year after discharge

	March 2015	May 2016
Anthropometric		
Weight in kg	75	72
BMI	28.2	27.7
Laboratory		
Hb in g/dL	12.7	15.3
MCV	103.8	98.8
Leucocytes as 10^3/μL	3.9	5.7
Platelets as 10^3/μL	120	165
INR	1.18	1.14
AST in U/L	33	31
ALT in U/L	41	34
GGT in U/L	145	165
AP in U/L	90	116
Albumin in g/dL	3.3	4.3
Bilirubin in mg/dL	1	1.3
Creatinine in mg/dL	0.59	0.5
Na in mmol/L	139	144
Child-Pugh	B (7)	A (6)
MELD	8	9
Ammonia in μmol/L	62	23
Thyroid function		
TSH in mUI/L	69.86	2.66
T4free in ng/dL	0.6	
PHES		
Symbol digit test as points	8	34
Number connection test A in s	78	25
Number connection test B in s	514	73
Serial dotting test in s	79	37
Line tracing test as seconds + errors	200	100
Final score	-9	1
Magnetic resonance spectroscopy		
Glx/Cr	1.884	0.439
Cho/Cr	0.934	1.204
mI/Cr	0.064	0.529

ALT: Alanine aminotransferase; AP: Alkaline phosphatase; AST: Aspartate aminotransferase; BMI: Body mass index; Cho/Cr: Choline/creatine; GGT: Gamma-glutamine transferase; GLx/Cr: Glutamate-glutamine/creatine; Hb: Haemoglobin; INR International normalized ratio; MCV: Mean corpuscular volume; MELD: Model for end-stage liver disease; mI/Cr: Myoinositol/creatine; T4free: free thyroxine; TSH: Thyroid-stimulating hormone.

Increasing levothyroxine dose resulted in steady clinical and laboratory improvements. At 1 wk later, the patient was discharged under levothyroxine 75 μgr/d, rifaximin 600 mg/12 h, lactulose and carvedilol 6.25 mg/12 h leading to clinical improvement and restoration of thyroid function in the following 4 wk. She had no admission or decompensations during the next months, rifaximin was removed and lactulose was used occasionally.

The patient was re-evaluated 1 year later. She was completely asymptomatic, fully active, back to work without mental complaints, as reported by her and her relatives. She had stable liver disease (Table 1), normal thyroid function and venous ammonia levels. Her performance at PHES improved dramatically in all the tests, with a final score within the reference population. Similarly, an improvement in her EEG was observed (Figure 1B). MRI showed no changes in the hyperintensity in basal ganglia on T1-weighted images (Figure 1D) but it showed an improvement in periventricular white matter hyperintensities on FLAIR images (Figure 1F). Single voxel proton MRS located in the right centrum semiovale (Table 1) showed a decrease in the glutamate-glutamine/creatine peak (Glx/Cr), together with an increase in the myoinositol/creatine (mI/Cr) and choline/creatine (Cho/Cr) peaks at short echo-time (23 ms).

We considered that currently there was no need for an intervention over the porto-systemic shunt. Her routine follow-up showed that she is clinically stable and asymptomatic under medical treatment (carvedilol 6.25 mg/12 h, levothyroxine 112 μg/d, sitagliptin 50 mg/d and lactulose occasionally).

DISCUSSION

Since hypothyroidism shares symptoms with HE, it is considered within the differential diagnosis of HE. However, there is scarce information on whether hypothyroidism may coexist and even precipitate HE and on how to attribute brain dysfunction to one or the other entity. The present report supports the role of hypothyroidism as a precipitating factor of HE by inducing hyperammonaemia and illustrates the difficulties in diagnosing HE when it coexists with other causes of brain dysfunction.

The lack of specific symptoms or laboratory tests that ensure the diagnosis of HE make therapeutic decisions sometimes difficult. A practical approach would be treating all the potential aetiologies of brain dysfunction, but this may not be the optimal strategy if it involves aggressive treatments with high risk of serious complications. Shunt embolization may be a good option in patients with HE secondary to portosystemic shunts, but such patients should be carefully selected since a rise in portal pressure with risk of variceal bleeding and ascites may occur.

The question, whether the neurological impairment was caused by the hypothyroidism or was attributable to the large portosystemic shunt, was very important to make further therapeutic decisions. Although both hypothyroidism and HE may present with mental slowness and memory impairment[1], asterixis and disorientation are more commonly observed in HE. Our patient's EEG pattern (slow activity and generalized triphasic waves) is characteristic of, although not specific for, HE[2] and has seldom been reported in hypothyroidism[3]. Furthermore, MRI depicted on T1-weighted images high signal in basal ganglia, which is frequently seen in patients with cirrhosis or portal-systemic shunts. Besides, FLAIR sequences showed high signal intensity in hemispheric white matter. This finding has been shown to improve after resolution of episodic HE or liver transplantation (LT) and has been attributed to mild brain oedema[4]. All these findings

Table 2 Clinical cases of patients with hypothyroidism and hyperammonaemia reported in the literature

Year	Ref.	Clinical presentation	Thyroid disease	Liver disease	Evolution
1992	Hitoshi *et al*[6]	Mild dementia, slow speech, hyperreflexia, dysmetria, asterixis with hyperammonaemia. Progression to coma despite lactulose treatment.	Hypothalamic hypothyroidism	Cirrhosis and portal hypertension	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia NRL disturbances
1999	De Nardo *et al*[7]	Hoarseness, fatigue, tongue swelling, myopathy. Hyperammonaemia.	Primary hypothyroidism	none	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia Systemic symptoms and myopathy
2000	Thobe *et al*[8]	Coma, hyperammonaemia. Unresponsive to lactulose.	Primary hypothyroidism	Compensated cirrhosis	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia NRL disturbances
2001	Yamamoto *et al*[9]	Dysarthria, disorientation.	Primary hypothyroidism	Decompensated cirrhosis	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia NRL disturbances
2007	Rimar *et al*[10]	Coma, hyperammonaemia. Unresponsive to lactulose.	Primary hypothyroidism	Compensated cirrhosis	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia NRL disturbances
2007	Khairy *et al*[11]	Grade Ⅲ hepatic encephalopathy.	Primary hypothyroidism	Decompensated cirrhosis	Thyroid hormone replacement improved: Hypothyroidism NRL disturbances
2012	Redkar *et al*[12]	Coma, hyperammonaemia. Unresponsive to lactulose.	Primary hypothyroidism	Decompensated cirrhosis	Thyroid hormone replacement improved: Hypothyroidism Hyperammonaemia NRL disturbances

support the diagnosis of HE. However, the parallelism between the time course of the cognitive impairment and the hypothyroidism, the lack of response to hypoammonaemic measures, and the neurological improvement following normalization of thyroid function implies a relevant role for hypothyroidism.

Ammonia is thought to have an important role in HE. In normal conditions, ammonia is mainly generated in the gut, then is extracted in the liver and eliminated as urea by the kidney. In liver failure and/or in patients with portal-systemic shunts, ammonia reaches the systemic circulation and the central nervous system[1]. Ammonia is metabolized to glutamine in astrocytes through the glutamine synthetase enzyme and both ammonia and glutamine exert their toxic effects on the brain. Muscle and kidney glutamine synthetase, together with kidney glutaminase, also regulate plasma ammonia levels and they are especially relevant in the context of liver failure. Patients with cirrhosis characteristically show an increase in Glx/Cr peak and a decrease of Cho/Cr and mI/Cr in MRS of the brain. Abnormalities in Glx/Cr have been interpreted as an increase in brain glutamine secondary to the ammonia detoxification in astrocytes, whereas disturbances in Cho/Cr and mI/Cr have been interpreted as a compensatory response to the increased intracellular osmolality caused by glutamine accumulation. The severity of these disturbances has been associated with HE, and they are reversible following successful LT[5].

A potential interaction between thyroid status and ammonia metabolism arose in our patient. Hyperammonaemia in patients with hypothyroidism has been seldom described over the last two decades, and only in sporadic cases reports[6-12] (Table 2). Six out of seven of these patients (86%) are patients with liver cirrhosis. All those cirrhotic patients exhibited clinical manifestations suggestive of HE (grade Ⅰ to Ⅳ), whereas the subject without liver disease had not. A common finding in all these cirrhotic patients with hypothyroidism was that neurological disturbances were refractory to hypoammonaemic therapy and improved with thyroid hormone replacement.

The mechanisms by which hypothyroidism may induce hyperammonaemia are not identified. In a rodent model, hypothyroidism was associated with increased urea cycle enzyme activity and decreased

glutamine synthetase in the liver[13]. However, a decreased urea synthesis rate was found in women with hypothyroid[14], suggesting a decrease in ammonia clearance *via* urea synthesis during hypothyroidism. We are not aware of data regarding a potential effect of thyroid hormone on muscle glutamine synthetase or gut glutaminase, which could also influence ammonia metabolism. A potential mechanism could involve thyroid hormone impact on glutamine synthetase activity not only in liver but also in muscle and kidney. A decrease in glutamine synthetase activity during hypothyroidism could contribute to hyperammonaemia that might revert with thyroid function restitution.

Hashimoto's encephalopathy, an autoimmune disease of the thyroid gland, may manifest as a wide range of neurological symptoms. It could lead to hypothyroidism and should be considered when evaluating patients with neurological disturbances refractory to standard therapy.

As in previous reports, our patient exhibited clinical manifestations suggestive of HE as well as hyperammonaemia refractory to hypoammonaemic measures, both of which reversed with thyroid hormone. Additionally, thyroid hormone replacement was also linked to an improvement in the periventricular white matter hyperintensities on FLAIR images and in MRS results, with a decreased in the Glx/Cr peak and an increase in the mI/Cr and Cho/Cr peaks. This novel observation supports that neurological disturbances were concomitant with hyperammonaemia and higher brain exposition to ammonia consistent with HE.

In conclusion, this clinical case shows that hypothyroidism is associated with hyperammonaemia and enhanced ammonia brain exposition. In agreement with the few cases previously reported, these data support that hypothyroidism may precipitate HE in cirrhotic patients by inducing hyperammonaemia and/or enhancing ammonia brain toxicity. Therefore, patients with cirrhosis and refractory HE should be evaluated for hypothyroidism, particularly if aggressive treatments are being considered.

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COMMENTS

Case characteristics

A 58-year-old woman with cognitive impairment consistent with persistent hepatic encephalopathy (HE) and portosystemic shunt refractory to standard treatment was referred to our hospital.

Clinical diagnosis

Refractory cognitive impairment consistent with HE that paralleled the course of post-ablative hypothyroidism.

Differential diagnosis

The main differential diagnosis was neurological disturbances secondary to hypothyroidism *vs* refractory HE secondary to portosystemic shunt. The clinical, laboratory, electroencephalograph (EEG) and magnetic resonance imaging findings, together with the clinical resolution following thyroid replacement, support the role of hypothyroidism as a precipitating event of HE.

Laboratory diagnosis

Laboratory tests demonstrated hypothyroidism, hyperammonaemia and alterations consistent with cirrhosis.

Imaging diagnosis

T1-weighted hyperintensities in basal ganglia and periventricular white matter high signals on FLAIR sequence, together with markers of brain ammonia exposition in magnetic resonance, supported the diagnosis of HE.

Treatment

The patient was refractory to standard HE treatment. Thyroid hormone replacement was associated with clinical resolution of the syndrome, together with normalization of plasma ammonia levels, neuropsychological performance, EEG and magnetic resonance markers of brain ammonia exposition.

Related reports

Very few case reports linked refractory neurological impairment in cirrhotic patients with hypothyroidism. This case shows an improvement in hyperammonaemia and brain ammonia exposition following thyroid hormone replacement, which was associated with clinical resolution of neurological impairment.

Term explanation

HE is a brain dysfunction that frequently affects patients with chronic liver failure. Its symptoms and signs are not specific and may be present in other causes of brain dysfunction.

Experiences and lessons

In cirrhotic patients, hypothyroidism may precipitate HE by enhancing ammonia brain toxicity.

Peer-review

This is an excellent case report describing an incidence whereby hypothyroidism was a precipitating factor that contributed to a recurrence of HE in a patient with liver cirrhosis. Overall, the report was very well written, with a clear description of the time line of events followed by a thorough discussion of the literature and known interactions between hypothyroidism and HE.

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CASE REPORT

Endoscopic occlusion with silicone spigots for the closure of refractory esophago-bronchiole fistula after esophagectomy

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Masaya Uesato, Tsuguaki Kono, Yasunori Akutsu, Kentarou Murakami, Akiko Kagaya, Yorihiko Muto, Akira Nakano, Mizuho Aikawa, Tomohide Tamachi, Hiroyuki Amagai, Takahiro Arasawa, Yasuhide Muto, Hisahiro Matsubara, Department of Frontier Surgery, Chiba University Graduate School of Medicine, Chiba 260-8677, Japan

Author contributions: Uesato M wrote the manuscript; Uesato M, Kono T, Akutsu Y, Murakami K, Kagaya A, Muto Y, Nakano A, Aikawa M, Tamachi T, Amagai H, Arasawa T, Muto Y diagnosed and treated; all authors discussed the results and commented on the manuscript.

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Abstract

A 65-year-old man with cT1bN0M0 stage I middle thoracic esophageal cancer underwent subtotal esophagectomy and gastric tube reconstruction through the posterior mediastinal route after preoperative carbon-ion radiotherapy and chemotherapy in a clinical trial. Anastomotic leakage occurred, but it spontaneously improved. At six months after the operation, he was rehospitalized with a cough and dysphagia. An esophago-bronchiole fistula and stenosis of the gastric tube were observed. He first underwent stent placement in the gastric tube. Two weeks later, the syringeal epithelium was burned by argon plasma coagulation after stent removal. Endoscopic occlusion was then performed for the fistula with two guidewire-assisted silicone spigots. Two weeks later, he was discharged on an oral diet, and he has not developed recurrence of the fistula or cancer for three years. This is the first report of endoscopic occlusion with a guidewire-assisted silicone spigot through the esophagus.

Key words: Endobronchial Watanabe spigot; Guidewire; Fistula; Leakage; Esophagectomy; Esophageal cancer; Endoscopic occlusion

Core tip: Anastomotic leakage, which is a complication of esophagectomy, sometimes causes a refractory

fistula. An esophago-bronchiole fistula (EBF) is a relatively rare but serious complication. We performed endoscopic occlusion using an endobronchial Watanabe spigot (EWS), a type of silicone bronchial blocker that is widely used in the respiratory field to treat pulmonary air leak, hemoptysis and bronchopleural fistula. We herein report a new and unique technique using the EWS to close a refractory EBF after esophagectomy. This report is the first to show that endoscopic occlusion using the EWS through the esophagus can simply and safely repair a refractory fistula.

Uesato M, Kono T, Akutsu Y, Murakami K, Kagaya A, Muto Y, Nakano A, Aikawa M, Tamachi T, Amagai H, Arasawa T, Muto Y, Matsubara H. Endoscopic occlusion with silicone spigots for the closure of refractory esophago-bronchiole fistula after esophagectomy. *World J Gastroenterol* 2017; 23(28): 5253-5256 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i28/5253.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i28.5253

INTRODUCTION

Anastomotic leakage, which is a complication of esophagectomy, sometimes causes a refractory fistula[1]. An esophago-bronchiole fistula (EBF) is a relatively rare but serious complication. Conventional therapy, such as hemoclipping, fibrin glue and stenting, are often performed[2-4]. However, surgical repair may be ultimately performed. We herein report a new and unique technique using an endobronchial Watanabe spigot (EWS) through the esophagus to close a refractory EBF after esophagectomy.

CASE REPORT

A 65-year-old man with cT1bN0M0 stage Ⅰ middle thoracic esophageal cancer underwent subtotal esophagectomy and gastric tube reconstruction through the posterior mediastinal route after preoperative carbon-ion radiotherapy and chemotherapy in a clinical trial. Anastomotic leakage occurred, but it spontaneously improved. There was no residual cancer in the resected specimen. At six months after the operation, he was rehospitalized with a cough and dysphagia. An EBF at the right B1 bronchiole and stenosis of the gastric tube were observed (Figure 1). The region of the anastomotic leakage and the region of EBF were same. The patient first underwent full-covered stent (Flexella-J; ELLA-CS, s.r.o., Hradec Kralove, Czech Republic) placement in the gastric tube under fluoroscopic guidance. Two weeks later, we recognized the release of the stenosis with the persistent EBF after the stent removal. Hemoclipping was performed for the fistula but failed. We next burned the syringeal epithelium by argon plasma coagulation (Figure 2). At the same time, endoscopic occlusion was performed. Under fluoroscopic guidance,

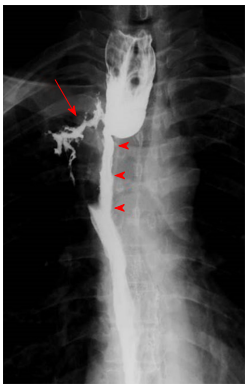

Figure 1 The radiographic image showed an esophago-bronchiole fistula from the anastomose to the right B1 bronchiole (arrow) and approximately 5 cm stenosis of the upper gastric tube (arrowhead).

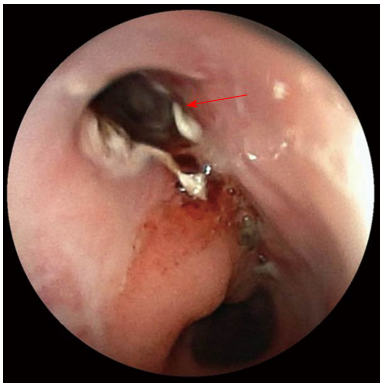

Figure 2 The epithelium of the esophago-bronchiole fistula was burned using argon plasma coagulation (arrow).

a 0.035-inch guidewire (Jagwire; Boston Scientific, Natick, MA, United States) was inserted into the EBF through an ultrathin endoscope (EG-580NW; FUJIFILM, Tokyo, Japan). After the endoscope was removed, a 5-mm endobronchial Watanabe spigot (EWS; Novatech, La Ciotat, France) was penetrated through its long axis using the guidewire (Figure 3A). After inserting a snaretube (MAJ-10; OLYMPUS, Tokyo, Japan) to the endoscope channel, the guidewire was retrogradely passed to the endoscope through the tube. Under fluoroscopic and endoscopic guidance, the EWS was wedged into the EBF (Figure 3B). Another 7-mm EWS was additionally inserted in a similar manner. The image just after the insertion showed the fistula occluded by the EWS (Figure 4A and B). Two weeks later, he was discharged on an oral diet, and he has not developed recurrence of the fistula or cancer for three years (Figure 5A-C).

DISCUSSION

A new and unique technique using a guidewire-assisted EWS through the esophagus proved useful for closing a refractory EBF after esophagectomy.

The patient in the present case had not only an EBF but also stenosis of the upper gastric tube. Upper

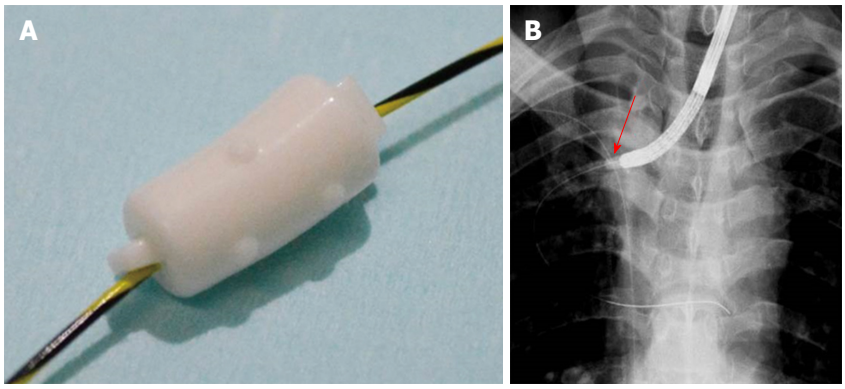

Figure 3 The endobronchial Watanabe spigot was penetrated through its long axis by the guidewire (A) (Push and Slide method[9]; B: Under fluoroscopic and endoscopic guidance, the endobronchial Watanabe spigot (arrow) was wedged into the esophago-bronchiole fistula.

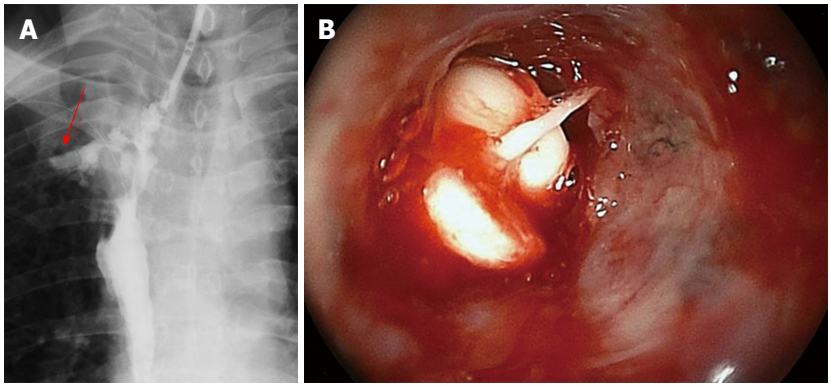

Figure 4 The radiographic image just after the insertion showed the fistula occluded by the endobronchial Watanabe spigot (A, arrow); B: In the endoscopic image just after occlusion, it was confirmed that the endobronchial Watanabe spigot is in the target fistula.

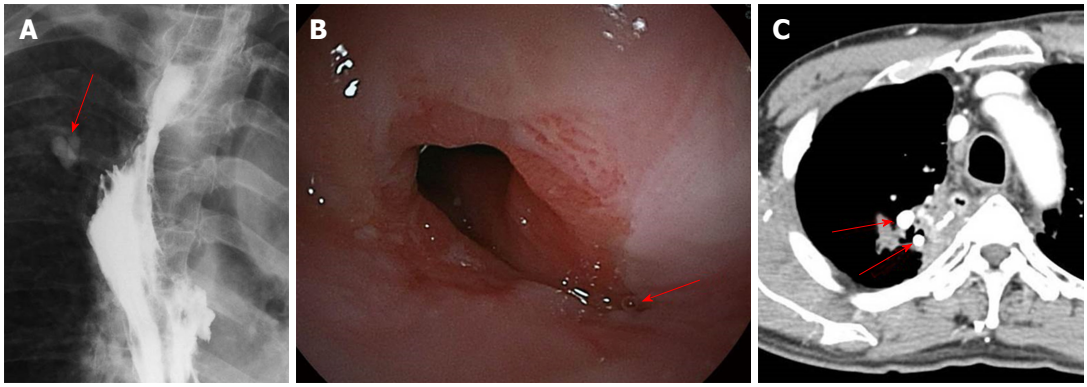

Figure 5 Since the endoscopic occlusion, the patient has not developed recurrence of the fistula for three years. Two endobronchial Watanabe spigots remain (A, arrow); B: In the endoscopic image passed for three years after occlusion, the fistula has disappeared; C: In computed tomography passed for three years after occlusion, two endobronchial Watanabe spigots remain (arrow).

gastric tube is the periphery of the blood flow, had approximately 5 cm stenosis. We therefore suspect that the cause of the fistula was the anastomotic leakage occurred by ischemia. A refractory fistula commuting with the airway system often causes pneumonia and respiratory failure. A number of minimally invasive treatments have been proposed for treating a refractory fistula, including hemoclipping[2], stenting[4], polyglycolic acid sheets and fibrin glue[3] and over-the-scope clipping[4]. In addition, since these therapies failed in the present case, surgery with a musculo-cutaneous flap was considered.

Before performing invasive treatment, we attempted endoscopic occlusion with an EWS, a type of silicone bronchial blocker[5] that is widely used in the respiratory field to treat pulmonary air leak[6], hemoptysis[7] and bronchopleural fistula[8]. We selected EWS occlusion through the esophagus to blockade the nearest portion of the EBF. The spigot is typically grasped with forceps and inserted into the affected bronchus using a flexible

bronchoscope[5]. However, because fistulas of the digestive system are more irregular than those in the tracheal route, it can be difficult to insert the EWS using forceps. Therefore, in the present case, a guidewire was first inserted, and then the EWS was placed along the guidewire.

In 2003, Miyazawa *et al*[9] first reported the "Push and Slide" method in Japanese. Recently, the usefulness of endobronchial occlusion for massive hemoptysis with a guidewire-assisted custom-made silicone spigot was reported[7]. We recommend this method, as the EWS can be inserted precisely. There are three kinds of size in the EWS (short axis/long axis: 5/8.5 mm, 6/10.5, 7/12.5)[6]. A fistula to the respiratory tract exceeding its size seems to be out of the indication. In an esophago-bronchial or tracheal fistula, the spigot may fall into the airway. Therefore, the best indication of the fistula could be occluded by the EWS is an esophago-bronchiole fistula has the length longer than approximately 10 mm. In addition, coagulating the syringeal epithelium by argon plasma coagulation before the EWS insertion might accelerate the occlusion of the EBF[2].

In conclusion, we successfully performed EWS occlusion through the esophagus for an EBF without complications. We therefore recommend EWS occlusion as a potential treatment for a refractory EBF after esophagectomy. This is the first report of the successful closure of a refractory fistula, although EWS occlusion through the gastrointestinal tract needs further exploration.

COMMENTS

Case characteristics

A 65-year-old man with esophageal cancer underwent subtotal esophagectomy and gastric tube reconstruction. At six months after the operation, he was rehospitalized with a cough and dysphagia.

Clinical diagnosis

An esophago-bronchiole fistula was observed.

Differential diagnosis

Aspiration pneumonitis.

Imaging diagnosis

An esophago-bronchiole fistula at the right B1 bronchiole and stenosis of the gastric tube were observed.

Treatment

He first underwent stent placement in the gastric tube. Two weeks later, the syringeal epithelium was burned by argon plasma coagulation after stent removal. Endoscopic occlusion was then performed for the fistula with two guidewire-assisted silicone spigots.

Experiences and lessons

A new and unique technique using a guidewire-assisted endobronchial Watanabe spigot through the esophagus proved useful for closing a refractory esophago-bronchiole fistula after esophagectomy.

Peer-review

The authors showed a new and unique technique using a guidewire-assisted endobronchial Watanabe spigot through the esophagus proved useful for closing a refractory esophago-bronchial fistula after esophagectomy. The technique is new and original. The text is well-written.

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