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

World Journal of *Gastroenterology*

World J Gastroenterol 2017 August 14; 23(30): 5451-5644

Published by Baishideng Publishing Group Inc

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

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2014-2017

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World Journal of Gastroenterology

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Weekly Volume 23 Number 30 August 14, 2017

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World Journal of Gastroenterology
Volume 23 Number 30 August 14, 2017

NAME OF JOURNAL
World Journal of Gastroenterology

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

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

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Jin-Lei Wang, Director
Yuan Qi, Vice Director
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World Journal of Gastroenterology
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
E-mail: editorialoffice@wjgnet.com
Help Desk: http://www.f6publishing.com/helpdesk
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PUBLISHER
Baishideng Publishing Group Inc
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-2238242
Fax: +1-925-2238243
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PUBLICATION DATE
August 14, 2017

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World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5451-5456

DOI: 10.3748/wjg.v23.i30.5451

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

EDITORIAL

Wilson's disease: Prospective developments towards new therapies

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Author contributions: Ranucci G, Polishchuck R and Iorio R contributed equally to the article's design, conception and writing.

Conflict-of-interest statement: All authors declare they have no conflicts of interest.

Manuscript source: Invited manuscript

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Received: February 3, 2017
Peer-review started: February 8, 2017
First decision: March 6, 2017
Revised: April 11, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: August 14, 2017

Abstract

Wilson's disease (WD) is an autosomal recessive disorder of copper metabolism, caused by mutations in the *ATP7B* gene. A clear demand for novel WD treatment strategies has emerged. Although therapies using zinc salts and copper chelators can effectively cure WD, these drugs exhibit limitations in a substantial pool of WD patients who develop intolerance and/or severe side effects. Several lines of research have indicated intriguing potential for novel strategies and targets for development of new therapies. Here, we review these new approaches, which comprise correction of *ATP7B* mutants and discovery of new compounds that circumvent *ATP7B*-deficiency, as well as cell and gene therapies. We also discuss whether and when these new therapeutic strategies will be translated into clinical use, according to the key requirements for clinical trials that remain to be met. Finally, we discuss the hope for the current rapidly developing research on molecular mechanisms underlying WD pathogenesis and for the related potential therapeutic targets to provide a solid foundation for the next generation of WD therapies that may lead to an effective, tolerable and safe cure.

Key words: *ATP7B*; Stem cell-derived hepatocyte like cells; Methanobactin; Heat shock protein 70; p38; JNK; Correctors; Translational medicine; Precision medicine

Core tip: Hepatocytes derived from human-induced pluripotent stem cells hold great promise for the drug discovery process, especially for Wilson's disease (WD). Therapeutic approaches offering correction of the *ATP7B* mutant function and/or less toxic suppression of copper accumulation are promising and could be available shortly. In particular, protein quality control components and their regulatory networks represent attractive new targets for WD-causing mutant correction. Cell and gene therapies, however, will require more studies before they can be considered for clinical trials. A key goal for WD advancement is international cooperation of specialized centers to overcome limited

availability of patients for study.

Ranucci G, Polishchuck R, Iorio R. Wilson's disease: Prospective developments towards new therapies. *World J Gastroenterol* 2017; 23(30): 5451-5456 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5451.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5451

INTRODUCTION

Wilson's disease (WD) is an autosomal recessive disorder of copper (Cu) metabolism, caused by mutations in the *ATP7B* gene, which encodes Cu-translocating ATPase expressed primarily in the liver[1]. *ATP7B* resides in the trans-Golgi membrane compartment and loads Cu onto newly synthetized apoceruloplasmin. In the case of Cu overload, *ATP7B* traffics to the canalicular membrane of hepatocytes and the associated cytoplasmic vesicular compartment, where it removes excess Cu through the bile. Therefore, all mutations that affect synthesis of *ATP7B*, its stability, correct localization in the trans-Golgi region and capacity of trafficking in the condition of intracellular Cu excess, determine toxic accumulation of Cu in hepatocytes, thereby leading to cell damage and subsequent release of Cu into the blood and ultimately affecting other organs[2].

The main clinical presentations of WD are liver and neuropsychiatric diseases. Existing therapies comprise treatment with either zinc salts or Cu chelators. While these drugs allow for sufficient control of the symptoms, they do not cure the disease. Additionally, chelators induce multiple severe toxicities, requiring discontinuation in approximately 30% of patients; these include hypersensitivity reactions, significant bone marrow suppression, degenerative changes in skin, nephrotoxicity and autoimmune disease[3]. Zinc therapy seems to fail in large cohorts of adult patients with liver disease when applied for very long periods, and its use in symptomatic Wilson's patients with liver disease remains controversial[4,5]. Generally, the psychiatric symptoms of WD are poorly controlled by available drugs[6]. Furthermore, an Italian study showed that, more than 36% of Italian pediatric WD patients responder to the available drugs, did not exhibit normalization of liver enzymes[7]. Finally, among adults with WD there are substantial rates of non-adherence to medication regimens (30%-50%), corresponding to both a high index of patients who suffer liver failure and who develop potentially irreversible neurologic or psychiatric symptoms[8].

Liver transplantation (LT) is a treatment option for the most severe (life-threatening) cases of WD. LT is a curative therapy, with biliary Cu excretion being restored, neurologic and psychiatric disease stabilizing or improving, and Kayser-Fleischer rings disappearing over time[9]. Unfortunately, LT does not represent a general therapeutic strategy, given the high rate of complications and the need for long-life immunosuppressive therapy[9]. Thus, there remains a clear need for novel treatment strategies aimed at curing WD through correction of the cellular defect.

During the last few years, the perspective on alternative WD therapeutic strategies has been completely revolutionized, as summarized in this review. And, the field of WD translational research and clinical management is currently experiencing an unexpected burst that hopefully will lead to an expanded range of therapies available to patients.

DISEASE MODELING

The four animal models of WD - the Long-Evans Cinnamon (LEC) rat, the inbred mouse models (toxic milk, the Jackson Laboratory toxic milk) and the *Atp7B* knockout (*Atp7b*$^{-/-}$) mouse - lose *ATP7B* function and manifest liver disease due to Cu accumulation[2]. Surprisingly, *Atp7b*$^{-/-}$ mice does not exhibit susceptibility to neurological disease and LEC rats and toxic milk mouse develop only a mild neurologic involvement. Yet, no mice models carrying the most frequent missense mutations of *ATP7B* (H1069Q or R778L) are available. On the other hand, the metabolic pathways of rodents are frequently vastly different from those of man, and this is the pivotal reason that efforts persist to develop a platform of human cells.

Hepatocytes obtained *via* liver biopsy cannot be considered as a good model system for WD studies. Indeed, it is extremely difficult to keep human hepatocytes from biopsies in culture, as they do not proliferate and rapidly undergo apoptosis. In addition, these cells are generally already damaged at the moment of liver biopsy execution.

The recent development of stem cell-derived hepatocyte-like cells (HLCs) has circumvented this problem and opens new avenues for gene- and/or drug-based therapy to treat liver diseases. This new "*in vitro* model" better mimics patient cell biology. HLCs can be fairly easily derived from induced pluripotent stem cells (iPSCs) that are generated through the reprogramming of human fibroblasts[10]. In this context, it would be particularly helpful to obtain HLCs from WD patients. Some steps have already been taken in this direction, with HLCs derived from patients with missense *ATP7B* mutation having been developed[11].

iPSCs harbor the same unique characteristics of embryonic stem cells, *i.e.*, they can be indefinitely expanded *in vitro* and differentiated in all cells types derived from the three germ layers. Thus, iPSC technology features the potential benefits of embryonic stem cells while addressing their major ethical and scientific concerns: embryo destruction and immune-incompatibility. In this manner, human iPSCs represent an unlimited source of human hepatocytes for

translational research and hold great promise for drug screening and liver disease modeling in particular. In addition to their HLC differentiation capacity, iPSCs can differentiate into neurons, providing a serious advantage in studying whether and how certain *ATP7B* mutations contribute to neurologic dysfunction in WD patients.

CORRECTION OF DYSFUNCTIONAL *ATP7B*

Alternative therapeutic approaches offering correction of *ATP7B* mutant function and/or less toxic suppression of Cu import have been investigated in recent years. It is worth noting that most of the *ATP7B* mutations belong to the missense (58%) or small deletion/insertion (27%) categories, thereby resulting in aberrant protein products that frequently exhibit residual Cu-transporting activity but which undergo strong degradation due to misfolding and retention within the endoplasmic reticulum (ER)[12]. This is the case for the most frequent *ATP7B* mutants, H1069Q (in 50%-60% of European and North American WD patients) and R778L (in 40% of East Asian patients). Thus, manipulating their translocation from the ER to ensure correct localization in the cell would be beneficial for a sizable portion of WD patients.

Correction strategy has been widely explored for the most frequent mutant in cystic fibrosis, the ΔF508 mutation of the cystic fibrosis transmembrane conductance regulator (CFTR)[13]. Like the *ATP7B* mutant, ΔF508-CFTR exhibits residual ion-transporting activity but undergoes strong retention and degradation in the ER. Considering these similarities, several labs have tested the potential of ΔF508-CFTR correctors, such as curcumin and 4-phenylbutyrate, for *ATP7B* mutant rescue. Both correctors were demonstrated as capable of reducing degradation of *ATP7B* mutants expressed in HEK293 cells[14]; however, curcumin failed to do so in HLCs expressing the R778L variant of *ATP7B*[11]. It remains unclear to what extent these drugs rescue localization and function of *ATP7B* mutant[14]. Curcumin has been reported to correct localization of *ATP7B*-R778L and to facilitate Cu efflux from patient-derived HLCs. However, in phase 1 clinical trials for CFTR correction, dietary curcumin was found to have very poor bioavailability and very low serum concentration[15]. Thus, the efficacy of this corrector for WD treatment remains questionable.

In order to identify new drugs and targets for *ATP7B* mutant correction, it will be important to first identify the quality control mechanisms that drive ER retention and degradation of the mutants. A few proteins (*i.e.*, COMMD1, clusterin) and peptides (*i.e.*, alpha-crystallin B peptides) control *ATP7B* mutants retention in the ER and/or their degradation[16,17]. However, these molecules do not belong to the "druggable" category and their potential for creation of drugs to cure WD remains largely unknown.

On the other hand, a recent analysis of global gene expression in hepatic HepG2 cell lines expressing either wild type *ATP7B* or *ATP7B*-H1069Q mutant revealed that suppression of p38 and JNK reduces retention of H1069Q and a few other *ATP7B* mutants in the ER, inhibits their degradation, and facilitates Cu excretion from mutant-expressing cells[18]. Therefore, p38 and JNK have become attractive targets for exploring approaches to mutant correction. Indeed, it has been recently reported[19] that p38 and JNK control a cluster of ER quality control genes that promote *ATP7B*-H1069Q degradation. This cluster comprises a well-known chaperone, heat shock protein 70 (HSP70), whose silencing protects the *ATP7B* mutant from degradation and facilitates its export from the ER to the Golgi complex where *ATP7B* normally works.

The recognition of HSP70 and its upstream regulators p38 and JNK as attractive new targets for *ATP7B* mutant correction has also elucidated their potential for normalization of Cu homeostasis in WD. Considering the importance of these proteins for several critical processes of the normal physiologic state, such as protein quality control, stress response, signal transduction and apoptosis, safe (condition-specific) inhibitors of HSP70, p38 and JNK must be identified in the context of preexisting drug-approved libraries to avoid significant impact on overall cell/organism health. Nonetheless, these new modulators represent a great opportunity for repurposing safe drugs for WD treatment.

NEW COMPOUNDS WITH THERAPEUTIC POTENTIAL

The current pharmacological treatments have largely failed in rescue of Cu homeostasis in WD patients with acute liver failure, leaving LT as the only viable option for treatment. A recent study using the LEC rat model of WD offered an extra option for such patients called methanobactin (MB), a peptide produced by *Methylosinus trichosporium* with an extremely high affinity for Cu[20]. Short-term MB treatment efficiently reversed acute liver damage due to Cu accumulation. This beneficial effect was associated with disposal of intracellular Cu, in particular from the mitochondria. Interestingly, the regular Cu chelators penicillamine and tetrathiomolybdate failed to clean toxic metal from the mitochondrial stores. As a consequence, MB treatment prevented hepatocyte death and the subsequent liver failure, elongating the life span of the LEC rat. Therefore this peptide seems to be a potential therapeutic agent for acute WD.

Findings from another recent study have suggested that liver X receptor (LXR)/retinoid X receptor agonist may be used to combat Cu toxicity in WD[21]. This approach does not require Cu chelation. Careful investigation of the transcriptional and metabolic changes in

samples from WD patients and *Atp7b*$^{-/-}$ mice revealed dysregulation of LXR as one of the key events in the pathogenesis of WD. Treatment with the LXR agonist T0901317 improved disease manifestations in the *Atp7b*$^{-/-}$ mice despite substantial Cu overload. Moreover, liver fibrosis and inflammation significantly decreased in LXR agonist-treated animals, while lipid profiles normalized and liver function and histology improved. Thus, the potential of T0901317 for WD cure is likely to be further explored.

CELL THERAPY

Cell therapy, as well as gene therapy, targets the liver, which does not express functional *ATP7B* protein, aiming to restore hepatobiliary Cu excretion[22]. Cell therapy in WD seems feasible because transplanted hepatocytes can integrate in liver parenchyma and restore deficient functions, including the transport of Cu into bile. Animal WD models, especially the LEC rat, has facilitated cell transplantation research in WD[22]. It was through this animal model that it was found necessary to repopulate less than half of the liver with healthy hepatocytes in order to achieve sufficient Cu removal and therapeutic efficacy. However, in that study, not every animal subjected to cell therapy showed equivalent benefits[22].

Extrahepatic cell therapy using engineering applications (*i.e.*, transplantation of liver tissue into small intestine or the abdominal cavity) is currently considered insufficient because Cu removal requires an intact bile excretion system. Thus, in the case of either cell or gene therapy in WD, liver is the first target considering the physiological restriction of *ATP7B* expression to hepatocytes as well as the availability of mechanisms to elminate Cu from the body[23]. Fortunately, transplantation studies using donor cells have confirmed the ability for biliary excretion, providing the first clue that biliary Cu transport is a feasible target for cell therapy in WD[24].

IPSC-derived HLCs can repopulate the liver but they support only some hepatocyte functions, which are restricted to fetal-like stages[10]. Therefore, transplanted cells can proliferate in the presence of native cells, having a low rate of proliferation. This happens when native liver cells have extensive DNA damage (as induced by toxins, ischemia and/or hepatectomy). Obviously these preconditioning regimens are undesirable in the setting of existing liver injury in WD. The ability of transplanted HLCs to express *ATP7B* was evaluated in liver of LEC mice, in which the transplanted cells did not increase in number over an observational period of several months[25]. Therefore, liver repopulation over a very long period implies that therapeutic correction in WD will require significant time[22].

GENE THERAPY

Gene therapy aims to correct the defect in native hepatocytes by providing healthy copies of *ATP7B* *via* introduction of a transgene by vectors capable of indefinitely integrating and/or persisting in cells. The proof-of-principle for gene therapy in WD came from adenoviral and lentiviral vector expression of *ATP7B* in the liver of rodent models, which achieved transient correction of Cu excretion and incorporation of Cu into ceruloplasmin[26].

Recently, these initial observations were confirmed with an adeno-associated vector serotype 8 (AAV8) encoding the human *ATP7B* cDNA placed under control of the liver-specific α1-antitrypsin promoter (AAV8-AAT-*ATP7B*). Expression of AAV8-AAT-*ATP7B* in *ATP7B*-deficient mice resulted in reductions of liver enzymes and recovery of physiological biliary Cu excretion[27]. This study showed a solid background for future translational studies. AAV-mediated gene therapy should allow *ATP7B* to be permanently expressed in WD patient liver and, hence, would eliminate a need for lifelong intake of Cu-reducing drugs. On the other hand, such risks as immune response, tumor biogenesis and poor integration into damaged cells[28] have to be weighted before AAV-mediated gene transfer therapy and will be a critical component of the repertoire of WD treatments. Furthermore, studies investigating the fate of genetically modified native cells within the liver in WD are still required[22].

TRANSLATING NEW THERAPIES TO PATIENT BEDSIDE

To establish whether and when these new therapies will be appropriate for clinical trials, a number of key requirements must be met. First, the particular reference population must be identified for treatment. It is likely that potential candidates for this population will include asymptomatic or presymptomatic WD patients with an early disease, people with liver failure or severe neurologic deterioration who could benefit from rapid removal of Cu, and those with severe adverse reaction to preexisting therapy[22]. Considering the potential latency of some treatments before they become effective and the severe risks linked to suspension of preexisting treatment in WD patients, it seems possible to consider the combination of existing drugs and new therapies to mobilize Cu for early clinical trials. Moreover, it will be important to define appropriate tests and effective endpoints that, in addition to clinical parameters, may be useful for demonstrating therapeutic efficacy, considering that drug monitoring necessitates use of noninvasive assays and biomarkers. In this context, the discovery of new biomarkers in WD will be of great significance to enable new effective treatment.

CONCLUSION

Emerging strategies for cure of WD foster new challenges that should be addressed by "precision" medicine, which takes in account the best available knowledge about disease mechanisms to establish approaches that yield an effective cure rate. Some compounds (tested in WD cell or animal models) seem to be close to translation into safe drugs and/or personalized treatments for WD patients. Advancing a WD cure will require an international cooperation to overcome the limited availability of affected patients who can be enrolled into clinical trials for development of new therapies. We hope that joint efforts between academia, industry, government and patient advocacy groups will allow for rapid progress and bring WD patient welfare to the forefront.

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P- Reviewer: Chen C, da Silva FP, Nemeth D **S- Editor**: Gong ZM
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5457-5459

DOI: 10.3748/wjg.v23.i30.5457

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

EDITORIAL

Are our endoscopy patients at risk for pyogenic liver abscess?

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Author contributions: All authors discussed the article and contributed to the writing of the comment; each of them approved the final manuscript.

Conflict-of-interest statement: The authors declare no conflict of interest for this editorial comment.

Manuscript source: Invited manuscript

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Received: May 25, 2017
Peer-review started: May 28, 2017
First decision: June 23, 2017
Revised: June 27, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: August 14, 2017

Abstract

This is an editorial comment on a recent publication reporting an increased rate of pyogenic liver abscesses (PLAs) after upper gastrointestinal panendoscopy. Its aim is to critically highlight the findings, limitations and potential clinical implications of this study. Issues of the mucosal barrier, the microbial flora, administration of antibiotics and underlying diseases are discussed. The probability of PLAs after endoscopies is not exactly known and the length of the "incubation period" remains unclear, but a possible causality should already suffice to make us think how to avoid them. Especially in patients with risk factors such as diabetes mellitus, end-stage renal disease, liver cirrhosis, biliary tract infection, and malignancies, the potential risk for PLAs should be considered. Unnecessary insufflation during endoscopy (causing mucosal stretching and microscopic tears) as well as mucosal damage (by direct abrasion with the scope) should be avoided in order to limit the invasiveness of the procedure as much as possible. And, in everyday routine, it should be kept in mind that in patients after endoscopy, especially in those with a breach of the mucosal barrier and significant comorbidities, PLAs can potentially develop and require timely administration of antibiotics as well as further diagnostic and therapeutic steps.

Key words: Endoscopy; Pyogenic liver abscess; Mucosal barrier; Gastrointestinal microbial flora; Comorbidities

Core tip: An increased rate of pyogenic liver abscesses after upper gastrointestinal endoscopy has been reported in a recent publication, leaving clinicians in some kind of predicament. Are we really exposing our endoscopy patients to a considerable danger? In an invited editorial comment on this study, the background, limitations and potential clinical implications of the findings are discussed.

Cerwenka H, Schemmer P. Are our endoscopy patients at risk for pyogenic liver abscess? *World J Gastroenterol* 2017; 23(30): 5457-5459 Available from: URL: http://www.wjgnet.

com/1007-9327/full/v23/i30/5457.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5457

INTRODUCTION

In the study published by Tsai *et al*[1] on 2135 patients with a first diagnosis of pyogenic liver abscess (PLA) and 10675 patients without PLA selected as reference controls, a higher rate of PLAs was found in those who had recently undergone an upper gastrointestinal panendoscopy. The authors concluded that clinical physicians should not ignore this. In spite of some limitations, especially related to the use of retrospective registry data, this study leaves us in some kind of predicament. Are we really exposing our endoscopy patients to a considerable danger?

MUCOSAL BARRIER

Theoretically, every endoscopy may cause a breach of the mucosal barrier and post-endoscopy bacteremia has indeed been reported[2-4]. However, PLA is known not to develop very easily and to affect predominantly patients with comorbidities and a weakened immune system[5,6]. In addition to the patient's condition, the extent of mucosal damage could also play a role. Are microscopic leaks relevant in immunocompetent persons? The study by Tsai *et al*[1] does not comprise the diagnoses made at the endoscopies. It would be important to know how many patients had pathologies leading to an obvious disruption of the continuity of the mucosal barrier like ulcers or erosive inflammation. Another point of easy entrance for bacteria causing PLA (in this case *via* the biliary route) would be the papilla of Vater after endoscopic papillotomy. Patients with recent ERCP were excluded in this study, but the authors provide no information on papillotomies made longer ago.

MICROBIAL FLORA

The mucosal surface of the gastrointestinal tract is colonized by 400 different bacterial species and subspecies[7]. In general, pathogens are part of a transient flora emerging under abnormal conditions, but some autochthonous germs may also become pathogenic under certain circumstances. The stomach, the duodenum and the jejunum contain only low numbers of microorganisms (103 to 104 bacteria/mL), notably acid-tolerant lactobacilli and streptococci, whereas the colon is the main site of microbial colonization[7]. It is, therefore, surprising, that, in this study, the risk of developing PLAs was higher for patients with upper gastrointestinal endoscopies than for those with colonoscopies. Likewise, a higher and not a lower rate of PLAs would have been expected in those with interventional endoscopic procedures associated with a more severe mucosal damage. As for the colonoscopies, the authors argue that the colon is further away from the portal venous and lymphatic circulations to the liver than the esophagus, stomach, and duodenum, and that it has a potent mesenteric lymphatic defense system[1]. Nevertheless, it is well known, that underlying pathologies in the whole gastrointestinal tract can be found in patients with manifest cryptogenic PLAs[8,9]. In terms of the interventional procedures, a possible explanation given, but not proven, by the authors is that more patients in these groups may have had antimicrobial treatment[1].

ANTIBIOTICS

In fact, the use of antibiotics is an essential point. Although they are not given routinely as a prophylaxis for endoscopy patients, some of the study patients will have been on current antibiotic regimens for various reasons. In general, PLAs respond well to the administration of antibiotics and the microbial spectrum of these abscesses is covered by many of them. For a clearer picture on the pathogenesis of the PLAs and of the role of antibiotics in this study, it would also be necessary to know the results of microbial cultures of the aspirates from the abscess cavities as well as details on the use of antimicrobial substances.

COINCIDENCES

Another issue requiring clarification is if some of the endoscopy patients already had concomitant incipient PLAs not yet diagnosed at the time of the examination. The presenting symptoms of PLAs may be vague and indeed, unspecific pain associated with undiagnosed PLA could even have been the motive for performing the endoscopy. In one of the study patients, the PLA was diagnosed as early as one day after the panendoscopy[1]. Here a coincidence seems most likely. The length of the "incubation time" for PLA after panendoscopy remains an unanswered question.

CONSEQUENCES

In conclusion, we do not exactly know the probability of PLAs after endoscopies, but a possible causality should already suffice to make us think how to avoid them. What can be done? It will not be advisable to renounce at endoscopies in patients requiring them for therapeutic, diagnostic or prophylactic purposes nor will it be reasonable to make these patients afraid. It will also still be indicated to do endoscopies in order to identify underlying diseases in patients with manifest cryptogenic PLAs[10] without fearing to aggravate the abscesses. However, especially in patients with risk factors such as diabetes mellitus, end-stage renal disease, liver cirrhosis, biliary tract infection, and

malignancies, the potential risk for PLAs should be considered. Strict disinfection processes are a matter of course. Unnecessary insufflation during endoscopy (causing mucosal stretching and microscopic tears) as well as mucosal damage (by direct abrasion with the scope) should be avoided in order to limit the invasiveness of the procedure as much as possible. And, in everyday routine, it should be kept in mind that in patients after endoscopy, especially in those with a breach of the mucosal barrier and significant comorbidities, PLAs can potentially develop and require timely administration of antibiotics as well as further diagnostic and therapeutic steps.

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P- Reviewer: Fujimori S, Sivandzadeh GR **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5460-5468

DOI: 10.3748/wjg.v23.i30.5460

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

REVIEW

Pancreatitis: Preventing catastrophic haemorrhage

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Conflict-of-interest statement: None of the authors has any potential conflicting financial interests relevant to this article.

Manuscript source: Invited manuscript

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Received: March 14, 2017
Peer-review started: March 18, 2017
First decision: April 5, 2017
Revised: May 3, 2017
Accepted: July 12, 2017
Article in press: July 12, 2017

Published online: August 14, 2017

Abstract

Pancreatitis represents nearly 3% of acute admissions to general surgery in United Kingdom hospitals and has a mortality of around 1%-7% which increases to around 10%-18% in patients with severe pancreatitis. Patients at greatest risk were those identified to have infected pancreatic necrosis and/or organ failure. This review seeks to highlight the potential vascular complications associated with pancreatitis that despite being relatively uncommon are associated with mortality in the region of 34%-52%. We examine the current evidence base to determine the most appropriate method by which to image and treat pseudo-aneurysms that arise as the result of acute and chronic inflammation of pancreas. We identify how early recognition of the presence of a pseudo-aneurysm can facilitate expedited care in an expert centre of a complex pathology that may require angiographic, percutaneous, endoscopic or surgical intervention to prevent catastrophic haemorrhage.

Key words: Complication of pancreatitis; Splenicartery; Pancreatitis; Haemorrhage; Pseudoaneurysm

Core tip: Pancreatitis represents nearly 3% of acute admissions to general surgery in United Kingdom hospitals. The presence of a fluid collection, necrosis and infection can directly contribute to such vascular complications which are associated with a significant morbidity and mortality. Early recognition of the presence of a pseudo-aneurysm can facilitate expedited care in an expert centre of a complex pathology that

may require angiographic, percutaneous, endoscopic or surgical intervention to prevent catastrophic haemorrhage.

Evans RPT, Mourad MM, Pall G, Fisher SG, Bramhall SR. Pancreatitis: Preventing catastrophic haemorrhage. *World J Gastroenterol* 2017; 23(30): 5460-5468 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5460.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5460

INTRODUCTION

Pancreatitis represents nearly 3% of acute admissions to general surgery in United Kingdom hospitals[1]. Although this may seem like a relatively small proportion of patients the impact of the pancreatitic patient has a significant influence on hospital services due to the sizeable morbidity and mortality associated with pancreatitis. The annual incidence of acute pancreatitis ranges from 13 to 45 per 100000 persons worldwide and is around 13-45 per 100000 people in the United Kingdom which is increasing at a rate of 2.7% each year[2-6]. The number of admissions due to pancreatitis in the US has doubled since 1988. There are now nearly 275000 admissions each year due to pancreatitis[7,8]. It is estimated that between 20%-30% of patients will have a further episode of acute pancreatitis and 10% of patients will go on to develop chronic pancreatitis[9-12]. The current incidence rates of chronic pancreatitis are around 5-23 per 100000 people[5,13,14]. Pancreatitis has a mortality of around 1%-7% which increases to around 10%-18% in patients with severe pancreatitis[1-4,15]. Patients at greatest risk were those identified to have infected pancreatic necrosis and/or organ failure[16].

In order to appropriately manage patients with pancreatitis multiple scoring systems have been created to try to determine the severity of pancreatitis and the risk of morbidity and mortality including the Glasgow score, Ransom score, Balthazar score, Apache 2 score, and the modified Marshall score[17-19]. Many continue to be used routinely in clinical practice to improve the treatment of patients with pancreatitis. Understanding that certain patients with more severe pancreatitis have a higher risk of morbidity and mortality allows clinicians to maintain a high index of suspicion in order to identify early the complications of pancreatitis. The revised Atlanta guidelines identify two key phases to acute pancreatitis. In early phase it is associated with systemic disturbances as the result of the host response to local pancreatic injury[17] (Table 1). The cytokine cascade as the result of pancreatic inflammation can be clinically manifested as a systemic inflammatory response syndrome which in turn may precipitate organ failure. The presence and duration of organ failure is a key determinant in defining severity of acute pancreatitis (Table 2). Late phase pancreatic inflammation is defined by the presence of ongoing systemic symptoms of inflammation such as persistent organ failure or local complications.

Local complications of pancreatitis often cause patients to develop a late phase pancreatitis. Interstitial oedematous pancreatitis with or without necrosis may be complicated by the presence of pancreatic and peri-pancreatic collections. The presence of such collections can be broadly divided into five categories. Acute peri-pancreatic fluid collections usually arise in the acute phase of pancreatitis and are confined with normal anatomical planes. They commonly remain sterile however if they persist for greater than 4 wk they commonly form into a pseudocyst. A pancreatic pseudocyst is formed beyond 4 wk and demonstrates a walled off fluid collection in the absence of necrosis. The presence of necrosis can be categorised as either an acute necrotic collection (ANC) or a walled off necrosis (WON). Infected necrosis may occur in either ANC or WON and will present with the presence of gas within the collection on imaging or positive microbiological samples taken directly from the area of necrosis (Table 3). Acute and chronic inflammation as a result of pancreatitis can cause both thrombotic and haemorrhagic complications. The presence of a fluid collection, necrosis and infection can directly contribute to such vascular complications which are associated with a significant morbidity and mortality.

ARTERIAL COMPLICATIONS IN ACUTE PANCREATITIS

Incidence, causes, and presentations

Vascular complications are relatively uncommon and occur in between 1%-23% of patients with pancreatitis. Venous complications are significantly more common than arterial complications. Arterial complications occur in around 1.3%-10% of patients[20-28]. Thrombosis of the portal and splenic vein has been shown to occur up to 23% and 22% of patients with pancreatitis respectively[23,29]. Portal vein thrombosis increases to 30% in those with peri-pancreatic collection and as high as 57% in patients with necrotising pancreatitis (Table 4)[29].

It is important to identify arterial complications early and treat aggressively as they are associated with a mortality of between 34%-52%[26,28,30-35]. Sixty percent of all acute haemorrhage in the presence of pancreatitis occurs as the result of ruptured pseudo-aneurysms in the presence of necrotising pancreatitis. Haemorrhagic pseudocysts without pseudoaneurysms and capillary, venous or small vessel haemorrhage only account for approximately 20% of cases[22].

Arterial complications often arise as the result of arterial wall disruption in the presence of free lipolytic and proteolytic enzymes. Severe pancreatic inflammation and necrosis leads to the disruption

Table 1 Early *vs* late pancreatitis

Early phase pancreatitis	Late phase pancreatitis
Systemic disturbances result from the host response to local pancreatic injury. Clinical manifestation with associated SIRS response. Usually lasts less than one week but may extend into the second week. Severity determined by presence of organ failure. Transient < 48 h. Persistent > 48 h.	Persistence of systemic signs of inflammation. Presence of local complications. Compensatory inflammatory response syndrome.

Table 2 Defining pancreatic severity

Mild acute pancreatitis	No organ failure No local complications
Moderately severe acute pancreatitis	Organ failure that resolves within 48 h (transient organ failure) and/or Local or systemic complications without persistent organ failure
Severe acute pancreatitis	Persistent organ failure (single/multiple) > 48 h

Table 3 Defining pancreatic and peri-pancreatic collections

Acute peri-pancreatic fluid collection	Don not have well defined walls Homogenous, confined to normal fascial planes in retroperitoneum May be multiple Likely to develop into a pseudocyst if they persist > 4 wk
Pancreatic pseudocyst	Fluid collection in peri-pancreatic tissues Occasionally partly/totally intra-pancreatic Well defined wall with essentially no solid material Occur typically after 4 wk
Acute necrotic collection	Fluid collection within the first 4 wk containing necrotic tissue and fluid. Presence of necrosis differentiates it from APFC
Walled off necrosis	Necrotic tissue contained within an enhancing wall of reactive tissue Usually occurs > 4 wk after the onset of necrotising pancreatitis
Infected necrosis	Presence of gas within collection Positive cultures post FNA

APFC: Acute peri-pancreatic fluid collection.

of pancreatic tissue and subsequent pancreatic fluid release which has a high enzymatic content. These disruptive processes are further proliferated by the cytokine cascade that develops due to pancreatitis. Such processes in combination with pressure necrosis can lead to pseudo-aneurysm formation or spontaneous arterial rupture. Bleeding may also develop from the disruption of the wall of a pseudocyst or WON.

The presence of radiologically inserted drains can also cause direct trauma to vessels. Drains can perpetuate local inflammation which in turn can further diminish arterial wall integrity. Surgical intervention in the form of necrosectomy can disrupt arterial integrity

Table 4 Different types and incidence of vascular complications in pancreatitis

Vascular complications of pancreatitis	Incidence
Arterial complications	1.3%-10% of patients with pancreatitis
Ruptured pseudo-aneurysm	60% of all acute haemorrhage in pancreatitis
Haemorrhagic pseudocysts without pseudoaneurysms	20% of all acute haemorrhage in pancreatitis
Capillary, venous or small vessel haemorrhage	20% of all acute haemorrhage in pancreatitis
Venous complications	1%-23% of patients with pancreatitis
Portal vein thrombosis	23% of patients with pancreatitis
Splenic vein thrombosis	22% of patients with pancreatitis
Superior mesenteric vein thrombosis	19% of patients with pancreatitis

and lead to the development of arterial complications. Multi organ failure, necrosis, peri-pancreatic infection, long-term anticoagulation and underlying vasculitis all increase the risk of vascular complications[35]. Pancreatic necrosis is specifically associated with an increased risk of major haemorrhage[28]. There is no evidence to confirm that fungal infections confer an increased risk of pseudoaneurysm formation in pancreatitis as compared to bacterial infection, however anecdotally in immunosuppressed patients fungal infections are closely associated with pseudoaneurysm formation. Due to the significant structural integrity of arteries the time taken for a pseudo-aneurysm to form can vary greatly from weeks to even years[22,29,36].

Although we are aware of certain risk factors for haemorrhage associated with pseudo-aneurysms it is challenging to quantify the impact that acute *vs* chronic inflammation has on vessel stability. Current evidence on acute haemorrhage is predominantly small volume case series of a mixed population of patients who have been treated for acute haemorrhage. However Zyromski *et al*[37] identified 24 patients with a pseudoaneurysm associated with pancreatitis of which 22 patients had acute on chronic pancreatitis and 2 patients presented during their first episode of acute pancreatitis. Twenty-one patients had a collection or pseudocyst and 2 had identifiable necrosis. Sethi *et al*[38] demonstrated that of those undergoing mesenteric angiography for bleeding associated with pancreatitis 8 had acute pancreatitis and 8 had chronic pancreatitis. Within the acute population three patients were diagnosed with necrotising pancreatitis and one required necrosectomy. Seven of the eight patients with chronic pancreatitis had confirmed pseudocyst formation and one developed acute necrosis on a background of chronic pancreatitis. Udd *et al*[21] looked at 33 patients with chronic pancreatitis and bleeding pancreatic pseudoaneurysms. They showed that 1.7% of admissions related to chronic pancreatitis

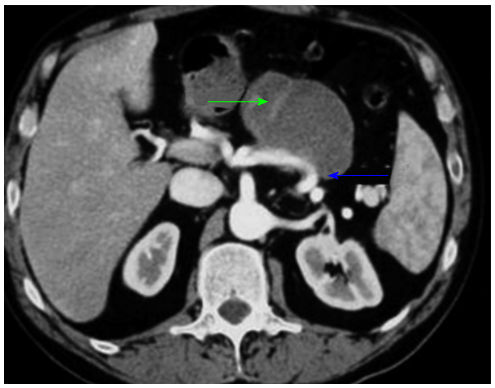

Figure 1 Computed tomography arterial and venous phase showing a pseudocyst (green arrows) eroding the splenic artery (blue arrows)[90].

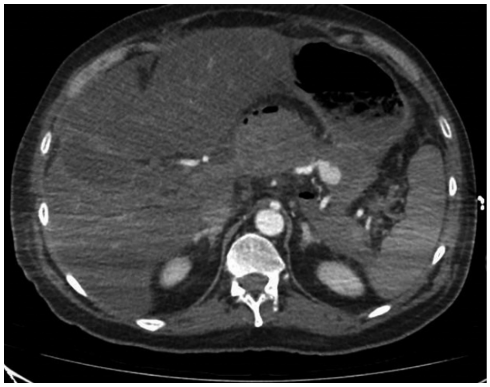

Figure 2 Computed tomography arterial and venous phases showing a pseudoaneurysm in a patient with necrotizing pancreatitis.

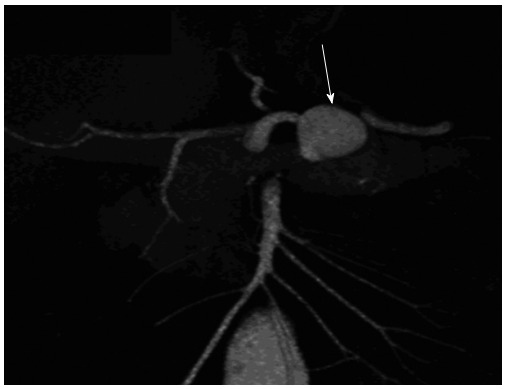

Figure 3 Three-D coronal maximum-intensity-projection computed tomography image in a 45-year-old man who had remote history of pancreatitis presented with back pain showing 2.0-cm pseudoaneurysm (arrow) arising from splenic artery[91].

were due to haemorrhage. They found that within the study population seven had pseudocysts two of which required percutaneous drainage and six patients had undergone a previous operation. There is no definitive evidence to show that patients with chronic pancreatitis and pseudocyst formation are at greater risk of developing arterial complications as compared to patients with severe acute pancreatitis with necrosis yet both are at high risk.

Symptomatic pseudo-aneurysms can present in varying ways however the most common symptom described is abdominal pain. Around 29.5% of patients will have abdominal pain which may reflect bleeding in the retroperitoneum. However there is a degree of ambiguity in the cause of such symptoms as pancreatitis in itself is painful. Bleeding into the gastrointestinal tract can occur in around 26.5% with haemosuccus pancreaticus (haemorrhage into pancreatic duct) being present in 20% of patients and also bleeding in pancreatic pseudocyst (Figure 1)[39]. Symptomatology is also dependent on the anatomical location of the pseudo-aneurysm.

Site of pseudoaneurysm

The most common site for pseudo-aneurysms is the splenic artery which occurs 35%-50% of occasions (Figures 2 and 3). The gastroduodenal and pancreaticoduodenal vessels each account for 20%-25% of pseudo-aneurysms. Mesenteric, colic and hepatic vessels commonly make up the remaining sites[20,31,40-50]. Aortic involvement can occur in up to 0.5% of cases[51,52]. Case reports have also identified individual cases of the left sub-capsular renal artery and the left phrenic artery[53,54]. Vessels in close proximity to the gastrointestinal tract have a greater risk of manifesting as luminal bleeding.

Imaging diagnosis

Identification of a pseudo-aneurysm is typically by CT imaging however the indication for imaging can vary[22,30,55]. Ultrasound has been shown to be less sensitive than CT in identifying such complications however, still has an important role in identifying venous complications such as portal vein thrombosis[29,56]. It may also have a limited role in those allergic to iodine and certain cases of renal insufficiency[57]. Current guidance for patients with complicated pancreatitis with evidence of significant inflammation, infection and/or organ dysfunction is to undergo planned cross-sectional imaging in order to determine whether there is a complication of pancreatitis. Cross-sectional imaging for pancreatitis should occur a minimum of 72 h after the onset of symptoms unless there is diagnostic ambiguity[58]. At present there is no strong evidence to differentiate between the use of CT and MRI however MRI may show greater detail in differentiating the different types of peri- and intra-pancreatic collections, such as necrosis with pus, necrosis without pus and fluid collection without necrosis; these findings may be better defined by MRI[17,59].

Present evidence supports contrast enhanced triple phase CT as the best imaging modality for vascular complications[34,60,61]. When a pseudo-aneurysm is identified subsequent management can be planned accordingly with the aid of both CT and angiographic guidance[62,63]. Due to the greater accessibility and

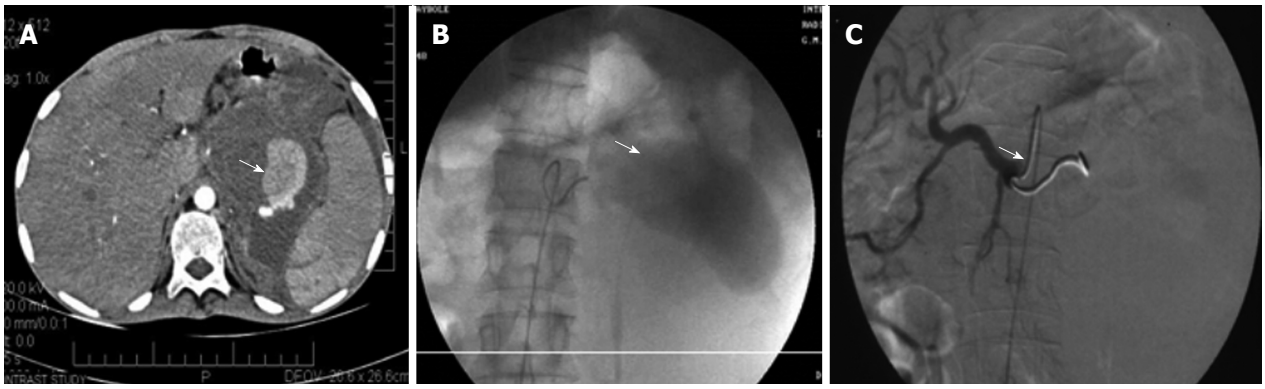

Figure 4 Splenic artery pseudoaneurysm before and after embolization. A: Post contrast computed tomography scan showing the pseudoaneurysm rising from the splenic artery; B: Pre embolization selective splenic arterial DSA angiography image showing pseudoaneurysm; C: Post embolization DSA image showing the coils inside the splenic artery with its resultant embolization[92].

improved vascular imaging triple phase contrast CT has become the imaging modality of choice in severe pancreatitis. Cross-sectional imaging may identify local pancreatic complications that may benefit from antibiotics, radiological intervention or very rarely surgery which in turn will aim to potentially speed the recovery of the patient. Less frequently pseudo-aneurysms will rupture causing acute haemodynamic compromise and emergency imaging is performed to determine the underlying cause. There is currently little evidence to specify the timing of follow up imaging and this should be guided by clinical assessment.

MANAGEMENT OF ACUTE HAEMORRHAGE

Historically the management of pseudo-aneurysm was primarily surgical however over the past two decades the first line intervention is now interventional radiology. Since there have been marked improvements in the field of interventional radiology the spectrum of pathologies that can be treated successfully has expanded greatly. In the United Kingdom the provision of interventional radiology services varies significantly and is concentrated in tertiary centres. This is particularly evident out of hours where few hospitals have a 24-h on call interventional radiology service. A study of the provision of services for acute upper GI haemorrhage has shown that of hospitals in the United Kingdom 46% were able to offer out of hours interventional radiology services whether that be at the hospital in question or *via* a network[64]. Acute haemorrhage in district general hospitals is therefore unlikely to be treated with interventional radiology and certainly the mortality of acute haemorrhage from pseudo-aneurysm secondary to pancreatitis may reflect this. Some patients in the acute setting may be suitable for transfer depending on haemodynamic stability. In a semi-elective setting appropriate patients can be transferred to regional centres with greater expertise in managing pseudo-aneurysms where they would have greater facilities to manage such complicated patients.

Trans-arterial embolisation of pseudo-aneurysms is commonly achieved by the placement of stainless steel or complex helical coils in the proximal and distal feeding vessel in order to isolate inflow and prevent back filling *via* collaterals (Figure 4). Haemostasis can be augmented by *N*-butylcyanoacrylate glue, ethiodised oil, gelfoam, thrombin, polyvinyl alcohol and other particles[21,37,38,41,43,65-69]. Increasingly in visceral aneurysm treatment covered stents have been used to exclude inflow to the aneurysm however they are less commonly used in the treatment of pseudo-aneurysms. Complications can arise both in failed and successful procedures. Access related complications might occur in the form of haematoma, dissection, pseudo-aneurysm and emboli. Complications may arise local to the pseudo-aneurysm including rupture, end vessel infarction, dissection and coil migration.

Mortality of pseudo-aneurysms secondary to pancreatitis has been shown to be between 34%-52%[26,28,30-35], however the literature shows significant improvement in case series of those who have undergone radiological intervention. It is important to note that a proportion of patients will have been too unstable for intervention and therefore this mortality may not be reported as widely in the studies of radiological or surgical intervention. Kim *et al*[65] in 2015 demonstrated that of 37 patients undergoing angiographic embolisation it was unsuccessful in only three patients. Two of whom re-bled from the primary pseudo-aneurysm and one developed a new pseudo-aneurysm. Of the 34 patients who underwent successful angiographic embolisation there were no episodes of re-bleeding with a mean follow up of 38 wk. Two patients died as a result of splenic abscesses and subsequent sepsis due to procedure related splenic infarction giving a mortality of 5%.

Udd *et al*[21] in 2007 identified 33 patients who were identified to have pancreatitis related pseudo-aneurysms at angiography however only 23 were suitable for embolization. Four of the 23 re-bled and

only three of those patients received successful repeat radiological intervention. Radiological intervention was successful in 22/33 patients (67%). The remaining 10 patients went on to require surgical cessation of bleeding. One patient who underwent interventional radiology and one patient who underwent surgery died giving a mortality of 6%. Bergert *et al*[20] 2005 identified 35 patients with bleeding secondary to pancreatitis related pseudo-aneurysms. Twenty six patients were identified to have pseudo-aneurysms at angiography however angiographic embolisation was the primary treatment in only 16. Two patients re-bled post-angiographic embolization. Nineteen patients required surgery to potentially stop bleeding. Three patients undergoing radiological intervention and four patients undergoing surgery died giving a mortality of 20%. The discrepancies in the number of patients undergoing radiological intervention and the mortality between Bergert *et al*[20] and Kim *et al*[65] may be reflected in the improvement of clinical practices from the related study dates of 1993-2004 and 2000-2012 respectively. Over the past two decades radiological provision, equipment and technical skills have improved significantly.

Tulsyan *et al*[68] in 2007 identified a mixed demographic of elective and non-elective patients (48 patients) with both visceral artery aneurysms and pseudo-aneurysms (28 patients) which showed marked success of radiological intervention. Forty seven of the 48 patients were embolised radiologically however three patients re-bled. Four patients died in the perioperative period all of whom were among the 22 patients who required urgent or emergent intervention because of hemodynamic instability giving a mortality of 18% in the non-elective group. Zyromski *et al*[37] in 2007 identified 37 patients with visceral pseudo-aneurysms 24 as the result of pancreatitis and 13 as the result of pancreatic surgery. Within the pancreatitis group 23 of the 24 patients underwent embolization and in each case it was successful at arresting haemorrhage. Initially in one patient a pseudo-aneurysm could not be identified at angiography but was confirmed after a repeat procedure. Twelve patients underwent surgery in an attempt to resolve the on-going pancreatic inflammatory process but not for treatment of a pseudo-aneurysm. On patient died as a result of a stroke unrelated to pseudo-aneurysm treatment.

Smaller studies assessing the management of pseudo-aneurysms secondary to pancreatitis have shown similar outcomes. Hsu *et al*[41] in 2006 (9 patients), Beattie *et al*[66] in 2003 (19 patients), Gambiez *et al*[67] in 1997 (14 patients) and Sethi *et al*[38] in 2010 (16 patients) identified that of a total of 58 patients radiological intervention was successful in only 35 patients (60%). Twenty patients subsequently underwent surgery (34%) and seven patients died (12%).

Angiographic embolisation of pancreatitis related pseudo-aneurysm has been shown to be very successful however there are occasions where the pseudo-aneurysm is inaccessible and therefore embolisation is not feasible. Increasingly in these circumstance image guided thrombin injection has been shown to have a role. The current literature identifies a number of case reports detailing successful embolization of a pseudo-aneurysm with percutaneous thrombin[62,70-84]. Of the 23 patients identified who underwent percutaneous thrombin injections four patients re-bled and required further intervention. Two patients had repeated thrombin injections, one underwent angiographic coiling and one is unknown. Other than re-bleeding no significant complications were documented. Evidence is increasing to show that percutaneous therapy is a viable alternative to angiographic therapy. The delivery of thrombin has been successfully used angiographically and percutaneously however in certain scenarios neither can provide appropriate access to allow for successful embolisation. Endoscopic ultrasound has been shown in case reports to provide appropriate access for thrombin injection of pancreatitis related pseudo-aneurysms. Both aneurysms of the gastro-duodenal and splenic artery have been embolised endoscopically without the need for re-intervention[85-89].

Surgery in patients with pancreatitis is known to be associated with significant morbidity and mortality which only increases in the presence of acute haemorrhage. With the improvement of minimally invasive techniques the role of surgery has diminished as angiographic, percutaneous and endoscopic techniques can appropriately exclude pseudo-aneurysms. However in spite of the technological advances in radiology and endoscopy, surgery will continue to play an important role in haemorrhage control where other techniques are not technically possible or available. There is currently no consensus on the surveillance of pseudo-aneurysms and the need for follow up. Patients with pancreatitis of a severity that leads to the formation of a pseudo-aneurysm will often undergo follow up imaging to ensure the resolution of local pancreatitis complications and in turn individually designed imaging follow up can be determined in coordination with the clinician and radiologist to ensure that the future re-bleed risks are mediated.

CONCLUSION

Pancreatitis has a number of aetiologies and many more potential complications which leads to it being a common pathology seen in every surgical department. Arterial complications of pancreatitis although rare are associated with a high morbidity and mortality. It is important to maintain a high level of suspicion for pseudo-aneurysm as they can be easily missed despite cross-sectional imaging. Early recognition of the presence of a pseudo-aneurysm can facilitate expedited care of this complex pathology that may require

angiographic, percutaneous, endoscopic or surgical intervention to prevent catastrophic haemorrhage.

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P- Reviewer: Du YQ, Fujino Y, Gonzalez-Ojeda A
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5469-5485

DOI: 10.3748/wjg.v23.i30.5469

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

MINIREVIEWS

Treating children with inflammatory bowel disease: Current and new perspectives

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Author contributions: Guariso G and Gasparetto M contributed equally to this work in respect to designing the review, contributing to the manuscript with intellectual content, writing the paper and final approval of the version to be published.

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

Manuscript source: Invited manuscript

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Received: February 26, 2017
Peer-review started: February 28, 2017
First decision: April 7, 2017
Revised: June 2, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: August 14, 2017

Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gut characterised by alternating periods of remission and relapse. Whilst the mechanism underlying this disease is yet to be fully understood, old and newer generation treatments can only target selected pathways of this complex inflammatory process. This narrative review aims to provide an update on the most recent advances in treatment of paediatric IBD. A MEDLINE search was conducted using "paediatric inflammatory bowel disease", "paediatric Crohn's disease", "paediatric ulcerative colitis", "treatment", "therapy", "immunosuppressant", "biologic", "monitoring" and "biomarkers" as key words. Clinical trials, systematic reviews, and meta-analyses published between 2014 and 2016 were selected. Studies referring to earlier periods were also considered in case the data was relevant to our scope. Major advances have been achieved in monitoring the individual metabolism, toxicity and response to relevant medications in IBD including thiopurines and biologics. New biologics acting on novel mechanisms such as selective interference with lymphocyte trafficking are emerging treatment options. Current research is investing in the development of reliable prognostic biomarkers, aiming to move towards personalised treatments targeted to individual patients.

Key words: Paediatric inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Treatment; Therapy; Immunosuppressant; Biologic; Monitoring; Biomarker

Core tip: This narrative review summarises the current practice in treating children with inflammatory bowel disease (IBD) and explores the new advances and future aims. A particular focus of the review are the peculiarities of the paediatric age in respect to the standard practice in adult patients with IBD. Whilst the cause of this condition remains only partly understood, a significant proportion of children does not respond to the treatment options currently available. Developing

new treatments is therefore a key target. Major advances have already been achieved in therapeutic drug monitoring.

Guariso G, Gasparetto M. Treating children with inflammatory bowel disease: Current and new perspectives. *World J Gastroenterol* 2017; 23(30): 5469-5485 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5469.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5469

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gut characterized by alternating periods of remission and relapse[1].

It comprises Crohn's disease (CD), ulcerative colitis (UC) and IBD-Unclassified (IBD-U), and its incidence has increased steadily worldwide, particularly in Western countries[2-4]. The disease affects both children and adults, with current estimated prevalence of 2.6 million people in Europe and 1.2 million in North America[5]. Approximately 25% of IBD patients are diagnosed before the age of 18[6]. Given that a cure for IBD hasn't been developed so far, treatments currently available are mainly aimed to induce and maintain remission. Therapeutic options include corticosteroids which have shown up to 80% efficacy in inducing remission in patients with CD. Other immunomodulators used for the treatment of IBD include thiopurines [*i.e.*, azathioprine (AZA) and 6-mercaptopurine], methotrexate (MTX) and biological treatments such as anti-tumour necrosis factor (anti-TNFa) therapies like infliximab (IFX) and adalimumab (ADA)[5].

CD is characterized by focal, patchy, transmural and granulomatous inflammation and it can affect any part of the intestinal tract as well as extra-intestinal tissue[4]. Peak age of diagnosis of CD is between 12 and 25[7].

Due to its typical onset in the young age and to the chronicity of the disease, medical treatment remains the cornerstone in CD, with most patients requiring lifelong therapy[4]. Whilst for CD surgery is generally an option restricted to patients resistant to maximised medical treatments or with specific complications, approximately 75% of patients with CD will eventually undergo surgical resection[4,8]. Nevertheless, according to the current epidemiology data available, up to 73% of these patients will experience endoscopic recurrence of disease at one year post surgery and 22% to 55% will have a clinical relapse at five years[8].

UC mainly involves the large bowel mucosa, with inflammation extending proximally from the rectum in a continuous fashion. In approximately 80% of children, the disease extent is proximal to the splenic flexure, whereas in adults it's more frequently left sided. Prolonged severe inflammation of the colonic mucosa is a known risk factor for the development of colorectal carcinoma[9].

Currently, proven medical treatment of paediatric UC is limited to a few options, including amino-salicylates, corticosteroids and thiopurines. More recently, anti-TNF agents have been established for the management of patients with UC who are refractory to conventional medical treatment[10]. Choice of therapy is mainly based on disease extension and severity of inflammation. Up to 25% of the total patients with UC currently require a colectomy because of ongoing, severe inflammation, unresponsive to medical therapy, or when the disease is steroid-dependent[11].

When a child is diagnosed with IBD, achieving early remission has a positive impact on normal child growth and development, long-term remission and quality of life, thus reducing the psychological burden[2]. Children tend to present with a more aggressive course of IBD, therefore immunomodulators and biological treatments are used extensively[2].

Achieving satisfactory nutritional status and reaching growth target should be one of the focuses in paediatric IBD. In fact, nutritional concerns are still common in children with CD (up to 65%-75% of cases) who are often underweight at presentation[3,6,12]. Even in the longer term, despite current treatment strategies for CD including biologics, short stature and slow growth are still encountered in paediatric CD.

Whilst an early diagnosis is pivotal to minimize growth deficiency, the signs of CD onset vary and can easily go unnoticed, causing growth deficiency and pubertal delay to precede the intestinal manifestations of the disease[12].

Poor bone health, delayed puberty, and growth failure may go on to complicate these patients' clinical management[2,6,12].

The pharmacokinetics (PK) and pharmacodynamics of drugs in children are different from those in adults and the approach to paediatric drug dosing needs to be based on the physiological characteristics of the child and the pharmacokinetic parameters of the drug[13].

Current European Medicines Agency (EMA) and United States Food and Drug Administration (FDA) guidance recommends that dose selection for paediatric studies is based on extensive and detailed prior information, starting with what has been learnt in adult populations[14]. Paediatric pharmacometric approaches are increasingly being applied to drugs already in use, but that remain unlicensed and off-label in children[14]. There are multiple factors contributing towards the pathogenesis of IBD, and the whole mechanism is yet to be entirely defined in its complexity. Current hypotheses suggest the host's genetic profile, immune system and environmental factors such as the gut microbiota as possible key factors[3].

IBD may develop from a chain of events involving alteration in the microbiome, increase in intestinal permeability leading to bacterial translocation, and

subsequent activation of the adaptive immune response to cause tissue damage (a model known as "bacterial penetration cycle hypothesis")[15].

As a result of the uncontrolled activation of the mucosal immune system, the pro-inflammatory cytokines released cause chronic inflammation of the gastrointestinal tract[5]. In consideration of the crucial role played by cytokines in the development of intestinal inflammation, all current treatments for IBD target downstream events in the host inflammatory response[15].

Given the complexity and heterogeneity of IBD, a holy grail of current research is to be able to customize therapy to a patient's predictive biomarker profile, in order to personalise treatment and to maximise response[11].

Studies in adult patients with CD have shown that treatments capable of inducing and maintaining endoscopic mucosal healing (MH) have a positive impact on the disease course, by reducing the number of clinical relapses, hospitalizations and surgical interventions[16]. Therefore, the current aim in the care of IBD for all ages is achieving intestinal MH, *i.e.*, beyond the simple resolution of symptoms[3].

Based on this evidence, the strategy of early introduction of immunomodulators and biological therapies ("top-down") to induce deep remission (long-term intestinal healing) is increasingly used in high-risk paediatric patients (*e.g.*, children with extensive disease distribution, severe perianal disease, no response to standard medical options, growth retardation and delayed puberty) as an attempt to modify the clinical course of the disease by inducing and maintaining remission, reducing hospitalizations, surgeries, use of corticosteroids, as well as promoting growth and pubertal development[3].

Based on the recommendations above, the conventional "step up" approach for paediatric CD, based on amino-salicylates, corticosteroids, and immunomodulators, is increasingly outdated for patients at high risk of complicated disease[17,18].

This narrative review aims to summarise the most recent advances in treating children with IBD and to provide with an overview of the new treatments in this field.

LITERATURE SEARCH

A Medline search using the keywords "paediatric inflammatory bowel disease", "paediatric Crohn's disease", "paediatric ulcerative colitis", "treatment", "therapy", "immunosuppressant", "biologic", "monitoring" and "biomarkers" was carried out.

Retrospective and prospective clinical studies, systematic reviews and meta-analyses published between 2014 and 2016 were selected for this narrative review. Studies conducted earlier were also taken into consideration whenever the data outline was considered relevant to the scope of the review.

DIET, MICROBIOTA AND FAECAL TRANSPLANT

Diet has an impact on the composition of the intestinal microbiome and gut immune status and currently there is growing evidence that the microbiota has a relevant role in the pathogenesis of IBD[15].

"Dysbiosis", an imbalanced intestinal microbiota with pro-inflammatory microorganisms prevailing on the protecting ones, has been repeatedly reported in patients with IBD[19].

Dietary interventions in children with active CD have proved evidence of a link between diet and the disease[15]. Exclusive enteral nutrition (EEN) using a polymeric formula for 6-8 wk, is the first-line therapy to induce remission in children with active CD[20,21]. EEN is effective in inducing remission in approximately 80% of patients, with a clinical remission rate similar to corticosteroids[3]. However, as opposed to steroids, EEN provides significant nutritional benefit and is superior in achieving MH[3].

EEN also leads to early MH, and long-term benefits of EEN-induced MH are currently being looked into[16]. Grover *et al*[16] conducted a prospective cohort study on 54 children with new diagnosis of CD, to evaluate the impact of early EEN-induced MH on predicting sustained remission (SR) on immunomodulators, without need for additional therapy like steroids, biologics or surgery. Paediatric CD Activity Index (PCDAI), C-reactive protein (CRP) and endoscopic assessment at diagnosis were paired with those post 6 wk of EEN. Complete MH was observed in 33%, and near complete in 19%. SR was superior in children with complete MH *vs* those with active endoscopic disease at 1, 3 and 5 years of follow-up, therefore the authors conclude that following induction of remission with EEN, complete MH is superior to clinical and biochemical remission in predicting SR over and beyond 3 years on maintenance immunomodulators[16] (Table 1).

Partial enteral nutrition with allowance of free diet hasn't been proved effective yet. It is unclear whether this depends on the supply of specific nutrients within the polymeric formula or on the exclusion of dietary factors during the course of exclusive polymeric diet[15].

Recent studies have aimed to evaluate which of the excluded dietary components in EEN may be responsible for the effect, in order to look into ways to allow a safe whole food diet[15].

Sigall-Boneh *et al*[15] validated a dietary intervention that allows whole foods but reduces exposure to dietary components that have been shown to induce inflammation, affect the microbiome and the mucous layer, increase gut permeability or the adherence and translocation of bacteria in mouse or cell line models.

Table 1 Summary of the cohort studies mentioned in the review including reference, study design and population, main results, conclusions

Ref.	Study design	Population	Main results	Conclusion
Nutrition				
Sigall-Boneh *et al*[15] *Inflamm Bowel Dis* 2014	Prospective cohort study	47 patients = 34 children + 13 young adults Mean age 16.1 ± 5.6 yr Active CD (PCDAI > 7.5 or Harvey-Bradshaw Index ⩾ 4)	Post treatment with 6-wk exclusion diet: access to specific foods + 50% of calories from polymeric formula Response in 37 (78.7%) Remission in 33 (70.2%) Decrease in CRP and ESR Normalisation of CRP in 70% of patients entering remission	Dietary therapy involving PEN with an exclusion diet seems to lead to high remission rates in early mild-to-moderate luminal CD in children and young adults
Grover *et al*[16] *J Crohns Colitis* 2016	Prospective cohort study	54 children with CD Age < 16 At least 6 wk EEN	Post EEN: Clinical remission (PCDAI < 10) in 45/54 (83%) Biochemical remission (PCDAI < 10, CRP < 5) in 39/54 (72%) Complete MH in 18/54 (33%) Nearly complete MH in 10/54 (19%) SR superior in children with MH *vs* active endoscopic disease: P 0.003 at 1 yr P 0.008 at 2 yr P 0.005 at 3 yr	Only complete MH post EEN induction predicts more favourable SR for up to 3 yr
Thiopurines				
Stocco *et al*[23] *World J Gastroenterol* 2015	Retrospective cohort study	12 paediatric patients = 6 CD + 6 UC	NAT1 genotypes (fast enzymatic activity) were associated with reduced TGN concentration The effect of NAT1 on TGN persists even 1 mo after the interruption of the aminosalicylate No effect of the NAT2 polymorphism was observed	NAT1 genotype affects TGN levels in patients treated with thiopurines and aminosalicylates and could therefore influence the toxicity and efficacy of these drug
Biologics and biosimilars				
Sharma *et al*[7] *Inflamm Bowel Dis* 2015	IMAgINE-1 study Phase-3, randomized, Multicentre, double-blind	Paediatric CD population = 192	Strong positive association between serum ADA concentration and disease remission/response to treatment Higher body weight, baseline CRP, lower albumin, previous treatment with anti-TNF and presence of anti-IFX antibody were associated with increased ADA clearance	Positive association between serum ADA concentration and remission/response in paediatric patients with moderate/severe CD
Nuti *et al*[18] *J Crohns Colitis* 2016	Prospective cohort study	37 biologic-naïve paediatric patients with CD	Biological therapy with IFX + AZA was effective in achieving MH (based on change in PCDAI and SES-CD) Combination of biologics + immunomodulators was more effective than biological monotherapy Improvement of mucosal lesions at 2 yr follow-up was predictive of favourable outcomes	Biologics improve mucosal lesions, more effectively if given in combination with immunomodulators. MH predicts a better disease course
Fumery *et al*[33] *J Pediatr Gastroenterol Nutr* 2015	Retrospective population based study (EPIMAD registry)	27 paediatric patients with CD experiencing IFX failure	Effectiveness and safety of ADA: Clinical benefit: 19 (70%) measured by the physical global assessment score Significant decrease in CRP in children responding to ADA (9 *vs* 15 mg/L) Cumulative probability of failure to ADA treatment: 38% at 6 mo, 55% at 1 yr Primary failure: 8 (30%) Secondary failure: 5 (26%) Adverse effects: 11 (40%)	Treatment with ADA was safe and effective in two-thirds of patients with pediatric-onset CD and IFX failure

Frymoyer *et al*[38] *J Pediatr Gastroenterol Nutr* 2016	Monte Carlo simulation analysis constructed using a published population pharmacokinetic model based on data from 112 children in the REACH trial	1000 simulated children	Trough IFX concentration > 3 mg/mL was achieved at week 14 in 21% for albumin level of 3 g/dL *vs* 41% for albumin of 4 g/dL	Standard IFX maintenance dosing in children with CD is predicted to frequently result in inadequate exposure, especially when albumin levels are low.
Dziechciarz *et al*[34] *J Crohns Colitis* 2016	Systematic review of 14 studies	Efficacy and safety of ADA I paediatric patients with CD	Pooled remission rates: At 4 wk: 30% (n = 93/309) At 3 mo: 54% (n = 79/145) At 4 mo: 45% (n = 18/40) At 6 mo: 42% (n = 146/345) At 8 mo: 57% (n = 20/35) At 12 mo: 44% (n = 169/383) Primary non-responders: 6% (13/207) Severe adverse events: 12% (69/599)	According to low-quality evidence based mainly on case series, approximately half of children with CD on ADA therapy achieve remission during the first year of the therapy with reasonable safety profile
Conrad *et al*[39] *Inflamm Bowel Dis* 2016	Observational, single-centre, prospective cohort study	21 paediatric patients (16 CD, 5 UC) with refractory IBD who had previously failed anti-TNFa therapy	Clinical response post treatment with vedolizumab: 6/19 (31.6%) at week 6 11/19 (57.9%) at week 22 Steroid-free remission in 1/20 (5%) at week 6, 3/20 (15%) at week 14 and 4/20 (20%) at week 22.	
Singh *et al*[44] *Inflamm Bowel Dis* 2016	Retrospective review on the experience with vedolizumab	52 paediatric patients with IBD, 90% of whom had failed ⩾ 1 anti-TNF agent	Week 14 remission rates: 76% for UC, 42% for CD, 80% of anti-TNF naïve IBD Week 22 remission rates: 100% anti-TNF naïve *vs* 45% anti-TNF exposed	Clinical response to vedolizumab in children with moderate/severe CD increases from week 14 to week 22
Sieczkowska *et al*[46] *J Crohns Colitis* 2016	Prospective cohort study	39 paediatric patients: 32 with CD, 7 with UC Children were switched from IFX originator to its biosimilar All patients had PCDAI ⩾ 25 at the time of switching	Clinical remission: 88% for CD 57% for UC	No differences in treatment efficacy, after switching from IFX originator to its biosimilar
Thalidomide				
Lazzerini *et al*[49] *JAMA* 2013	Double-blind, placebo-controlled, randomized clinical trial	56 padiatric patients with active CD, randomised to receive either thalidomide or placebo Almost all had not responded to thiopurines and 35% had not responded to biologics	Clinical remission achieved by 13/28 (46.4%) of the children treated with thalidomide *vs* 3/26 (11.5%) of those who received placebo (P = 0.01) Responses were not different at 4 wk, but greater improvement was observed at 8 wk in the thalidomide group [75% response in 13/28 (46.4%)] *vs* 3/26 (11.5%)(P 0.01) Of the non-responders to placebo who began receiving thalidomide, 11 of 21 (52.4%) subsequently reached remission at week 8 (P = 0.01). Overall, 31 of 49 children treated with thalidomide (63.3%) achieved clinical remission Mean duration of clinical remission in the thalidomide group was 181.1 wk *vs* 6.3 wk in the placebo group (P < 0.001).	Thalidomide compared with placebo resulted in improved clinical remission at 8 wk of treatment and longer-term maintenance of remission.
Lazzerini *et al*[50] *Inflamm Bowel Dis* 2015	Multicenter, double-blind, placebo-controlled, randomized clinical trial	26 paediatric patients with active UC, randomised to receive thalidomide or placebo All patients had had thiopurines and 35% had received prior IFX treatment	Clinical remission at week in 10/12 (83.3%) of the children treated with thalidomide *vs* 2/11 (18.8%) of those who received placebo (P = 0.005) Of the non-responders to placebo who were switched to thalidomide, 8 of 11 (72.7%) subsequently reached remission at week 8 (P = 0.01) Clinical remission in the thalidomide group was 135 wk compared with 8 wk in the placebo group (P < 0.0001).	Thalidomide compared with placebo resulted in improved clinical remission at 8 wk of treatment and in longer term maintenance of remission.

New treatments				
Tew *et al*[11] *Gastroenterology* 2016	Retrospective analysis of two cohorts: 1. phase 2 placebo-controlled trial; 2. observational study at a separate site	110 patients with UC (cohort 1) and 21 patients including UC and controls (cohort 2)	Increased expression of T-cell associated genes in baseline biopsies of anti-TNF naïve patients who achieved clinical remission in response to etrolizumab Patients with high colonic integrin aE expression showed greater benefit GZMA expression was different post-treatment	Levels of GZMA and ITGAE mRNAs in colon tissues can identify patients with UC who are most likely to benefit from etrolizumab GZMA is a promising biomarker for etrolizumab response
Sandborn *et al*[51] *N Engl J Med* 2016	Double-blind, placebo-controlled, phase-2 trial	197 adult patients with moderate-severe UC	Clinical remission at 8 wk: 16% of patients who received 1 mg of Ozanimod *vs* 14% who received 0.5 mg *vs* 6% of those who received placebo Clinical remission t 32 wk: 21% *vs* 26% *vs* 6% respectively Drop in absolute lymphocyte count at week 8: 49% from baseline in the group who received 1 mg of Ozanimod 32% from baseline in the group who received 0.5 mg	Ozanimod at a daily dose of 1 mg resulted in a slightly higher rate of clinical remission of UC than placebo
Allez *et al*[52] *Gut* 2016	Randomised, double-blind, parallel group trial	78 adult patients with CD Age 18-75 Disease duration ⩾ 3 mo CDAI 220-450 CRP ⩾ 10 mg/L Endoscopic evidence of inflammation	No significant difference in change in CDAI from baseline to week 4, between NKG2D group and placebo group Significant difference in change in CDAI at week 12 (delta CDAI -55, $P \leqslant 0.1$) between NKG2D group and placebo group Significant improvement noted in the non-failure to biologic subgroup treated with anti-NKG2D from week 1	A single s.c. dose of 2 mg/kg anti-NKG2D did not reduce disease activity at week 4 *vs* placebo, but the difference was significant at week 12

ADA: Adalimumab; CD: Crohn's disease; CDAI: Crohn's disease activity index; CRP: C-reactive protein; EEN: Exclusive enteral nutrition; GZMA: Granzyme A; IFX: Infliximab; ITGaE: Integrin aE gene; MH: Mucosal healing; NAT: N-acetyl transferase; PCDAI: Paediatric Crohn's disease activity index; PEN: Partial enteral nutrition; s.c.: Subcutaneous; SES-CD: Simple Endoscopic Score for Crohn's Disease; SR: Sustained remission; TGN: Thioguanine nucleotides; UC: Ulcerative colitis.

They recruited 47 patients including 34 children and 13 young adults with active disease, who were treated with a 6-wk exclusion diet that allowed access to specific foods and up to 50% of dietary calories from a polymeric formula.

The diet consisted in elimination of or reduction in animal fat, dairy products, gluten, and emulsifiers whereas fibre from fruits and vegetables was allowed. Clinical response was observed in 78.7% and remission in 70.2%, alongside improvement in CRP (normalized in approximately 70%) and ESR. On the basis of these results, the Authors recommend the use of this elimination diet in patients with mild-moderate disease, as it allows access to specific foods improving palatability and compliance[15] (Table 1).

So far, efficacy of microbiome-based therapies like probiotics or antibiotics has been limited in IBD[19]. Nevertheless, the recent focus on dysbiosis as a plausible key factor in IBD pathogenesis, has led to a growing interest in faecal microbiota transplantation (FMT) as a novel potential treatment option in IBD.

FMT is the administration of faecal material from a donor into the intestinal tract of a recipient, with the aim to change the microbiota composition and restore mucosal health. Over the past few years, FMT has been used successfully for the treatment of recurrent Clostridium difficile infection (CDI) (efficacy of 80%-95%), and is now being evaluated in other diseases possibly driven by the microbiota, including IBD[19].

There are several studies and case reports on the use of FMT in UC, but only two randomized control studies published to date[19,20,22].

The success rate of FMT in treating UC has been much more limited (clinical remission in 35%) in respect to its success in treating recurrent CDI. However, studies so far have been small and heterogeneous, therefore clear conclusions are difficult to make[19].

Even less data is available for FMT in CD, with only isolated cases or heterogeneous small series reporting overall clinical remission in 60%-75%[19].

At present, evidence on FMT in IBD is not strong

enough to recommend its use as part of routine treatment. Preliminary results are promising and more studies are needed to define the best indications, optimal timing, frequency, mode of delivery, and the most appropriate donor for each patient[19].

AMINOSALYCILATES

5-aminosalicylic acid (mesalazine, 5-ASA) acts topically on the gastrointestinal mucosa, with minimal systemic effect. Even though its exact mechanism of action is yet to be understood in its complexity, the pathways that are known to be involved include the blockade of IL-1 production and TNF-α receptor, the inhibition of cyclooxygenase and 5-lipoxygenase, and the blockade of the pro-inflammatory prostaglandin E2 and leukotrienes. On top of the inhibition of multiple inflammatory pathways and the suppression of the nuclear factor kappa B as a main result, amino-salicylates also possess potent anti-oxidant and free-radical-scavenger properties[4]. Amino-salicylates are mainly used in the induction and maintenance of remission in UC[23].

In CD, their use is no longer recommended in view of limited efficacy. However, there are studies suggesting a possible role in the postoperative maintenance of remission, as well as in the subgroup of children with mild, localised ileal disease. In addition, adult studies suggest a protective role of 5-ASA in IBD against colon cancer in patients with colonic location[23].

Given that sulfasalazine is not tolerated in 30%-40% of patients, particularly slow acetylators, the use of its therapeutically active component 5-ASA has led to the development of new formulations, that deliver higher concentrations of 5-ASA without the dose-limiting side effects of sulfasalazine. A wide range of these formulations is available and comprises pH-dependent release coated drugs (targeting the ileum), time-dependent release micro-granules enclosed within a semi-permeable membrane of ethyl-cellulose (released in the whole small and large intestine), and azo-bonded formulations released throughout the colon[4].

CORTICOSTEROIDS

Corticosteroids are used as first-line therapy for induction of remission in UC, particularly in patients with non-response to 5-ASA or with severe presentation, as well as to induce remission in CD when EEN is not possible[3,9,24].

The mechanism of action of corticosteroids consists in inhibiting protein synthesis and transcription, which ultimately results in down-regulation of pro-inflammatory cytokines, such as NF-kappa B, TNF-α, interleukin-1 and interleukin-6[9].

Clinical remission rates for CD are up to 80%, similarly to EEN, whereas MH rates are significantly lower[3]. Corticosteroids improve rapidly and effectively the signs and symptoms of disease in CD, however they are ineffective, and inappropriate, for maintenance therapy[17].

In children with moderate-severe active luminal CD, oral prednisolone is given at 1 mg/kg, with a maximum of 40 mg/d, followed by a weaning course over 8-10 wk. Intravenous steroids may be initially needed for severe disease and include methylprednisolone (1-1.5 mg/kg, maximum: 60 mg/d) and hydrocortisone (2-4 mg/kg per dose, maximum 100 mg/dose, four times a day)[3].

Adverse effects like adrenal suppression, growth failure, cosmetic and behavioural effects, are dependent on dose and duration[3].

Budesonide (maximum of 12 mg/d, followed by weaning course over 2-4 wk) is a topically acting corticosteroid with high first pass hepatic metabolism, which reduces the likelihood of adverse effects[3,9].

Budesonide is particularly recommended for patients with mild to moderate CD involving the distal ileum and/or right colon, as it has been shown to be non-inferior to conventional oral steroids for inducing remission in this specific group. It has also proved to be an effective therapeutic option as enema formulation for distal UC[9].

There are currently three formulations of budesonide: two standard formulations including a controlled-release capsule and a pH-dependent capsule both designed to target the ileum and right colon; and a more recent Budesonide-MMX® capsule that releases the drug throughout the entire colon[9].

THIOPURINES

Thiopurines are purine analogues used for the maintenance of disease remission in patients with CD and UC; they include the prodrug AZA and the antimetabolite 6-MP[25,26].

AZA is non-enzymatically degraded to 6-MP which is then metabolised to its active component, 6-thioguanine nucleotide (6-TGN)[8]. 6-TGN inhibits the proliferation of T and B lymphocytes, which results in a decrease in the numbers of cytotoxic T cells and plasma cells[8].

These dugs are able to block the rapid cell proliferation involved in inflammatory processes, which results in immunosuppression[26].

For the treatment of IBD, thiopurines are used at relatively low dosages, so their anti-inflammatory effect is mainly produced by the inhibition of the small GTPase Rac1, leading to apoptosis of activated T-lymphocytes. When given at higher dosages, as in oncological treatments, thiopurines mainly inhibit DNA synthesis[26].

Thiopurines are steroid sparing agents and have been proven effective maintenance treatment in paediatric IBD: studies have shown significantly lower cumulative steroid doses and relapse rates at 18 mo in children on 6-MP compared with placebo (9% *vs* 47%) as well as a reduced need for surgery in CD[3,23]. Recommended doses are 1.0 to 2.5 mg/kg per day for AZA and 1 mg/d for 6-MP[25].

Thiopurines are also used effectively to maintain surgically-induced remission in CD, even though a systematic review by Gordon *et al*[8] pointed out that the results for efficacy outcomes between thiopurines and 5-ASA in this group of patients are uncertain.

Testing the activity of the enzyme thiopurine-S-methyltransferase (TPMT) is recommended to guide thiopurine dosing avoiding adverse events[26].

Genetic polymorphisms in the TPMT gene are associated with a reduced enzymatic activity and an increased production of the active metabolites TGNs[23].

A large prospective study with 1000 individuals established TPMT activity reference intervals, with normal activity associated with a level $\geqslant$ 25 nmol/h per gram Hgb[27].

According to the current data available, 1 in 300 patients have a very low to absent TPMT activity (homozygous mutant TPMT), 11% have intermediate TPMT activity (heterozygous) and 89% have normal to elevated activity (homozygous wild type TPMT)[27].

The use of thiopurines is limited by an extensive spectrum of adverse events in up to almost half of patients, particularly within the first twelve months of treatment. Adverse effects include myelotoxicity, hepatotoxicity, pancreatitis and gastrointestinal complaints[26].

Measuring TPMT activity levels (phenotype) or determining TPMT genotype before initiating thiopurine therapy, is recommended by the FDA to limit the likelihood of side effects[27]. However, it still remains possible to develop significant myelotoxicity despite normal TPMT activity.

The use of TPMT activity and 6-TGN level monitoring has the potential to avoid nearly a quarter of episodes of myelosuppression[27]. In addition, 6-TGN monitoring is helpful to detect non-compliance, under-dosing, and drug resistance or refractory states[27].

Blood levels of thiopurine metabolites correlate with the efficacy and toxicity of these drugs as follows: TGN levels higher than 235 pmol/8 $\times$ 10^8 red blood cells reflect an adequate therapeutic level, whereas methyl mercaptopurine nucleotides levels above 5700 pmol/8 $\times$ 10^8 red blood cells are indicative of hepatotoxicity[23].

AZA or 6-MP can be started at the full recommended dose of 2-2.5 mg/kg per day or 1-1.5 mg/kg per day, respectively, in patients with normal to high TPMT activity level. Patients with an intermediate TPMT activity should start, instead, with a daily dose reduced by 30%-70%. Alternative therapy should be offered to individuals with low or absent TPMT activity, or they should be started at 10% of the suggested dosing, given three times per week[27].

The results of a recent retrospective study by Benmassaoud *et al*[27] evaluating the safety and efficacy of starting thiopurines at low dose *vs* full dose in adult patients with CD and normal TPMT, suggest that AZA should be started on full dose in patients with normal TPMT, rather than starting on a lower dose and increasing slowly. This is mainly due to the fact that patients with normal TPMT level may still be exposed to side effects that are unrelated to the enzymatic activity[27]. Overall, almost half of the adults treated with thiopurines discontinue their treatment due to ineffectiveness or intolerance[26]. It has been hypothesized that prescribing thioguanine (TG) therapy instead of AZA/6-MP reduces the release of potentially toxic metabolites, as its metabolism is less complex with a more direct conversion to the therapeutically active metabolite 6-TGN[1].

In a systematic review, Meijer *et al*[1] describe how TG therapy can represent a valuable option in adult IBD patients intolerant to conventional thiopurine therapy, with efficacy in 65% of patients and short term adverse events in 20%. However, TG is currently only used as experimental or rescue therapy and should not be used outside highly controlled situations[1]. Thiopurines and amino-salicylates are often used in combination in the treatment of IBD[23].

An increase in mean TGN blood levels has been reported in patients on concomitant treatment with thiopurines and 5-ASA. Moreover, a higher rate of myelotoxicity was observed in this group of patients compared with those treated with the thiopurine alone[22]. A plausible explanation comes from *in vitro* studies showing that amino-salicylates and their metabolites can inhibit the activity of TPMT, even though this observation has not been yet replicated *in vivo*[23].

The enzymes N-acetyltransferases (NAT1 and NAT2, EC 2.3.1.5) are responsible for the N-acetylation of multiple drugs including the amino-salicylates. Subjects are classified as rapid, intermediate or slow acetylators based on the activity of NAT1 and NAT2 that is genetically determined[23].

Stocco *et al*[23] evaluated the variation of the level of TGN after 5-ASA cessation and the role of genetic polymorphisms of NAT 1 and 2, in a group of 12 children recruited at two tertiary level paediatric gastroenterology units (6 CD and 6 UC) (Table 1).

Rapid acetylators with NAT1 genotypes were found to have reduced TGN concentration, and the effect of NAT1 activity on TGN persisted up to one month after discontinuation of the 5-ASA. NAT2 polymorphism, instead, did not produce any effect. These results, though limited by the small number of patients, show that the NAT1 genotype affects TGN levels in patients treated with thiopurines and 5-ASA and it may therefore impact on the efficacy and toxicity of these drugs[23] (Table 1).

MTX

MTX, a dihydrofolate reductase inhibitor, has long been used effectively to treat rheumatoid arthritis until it was brought into the field of IBD to treat patients with

refractory CD. Nowadays, it has become one of the principal alternatives to thiopurines as maintenance treatment[28,29].

Efficacy of MTX, given at 15 mg/m^2 once a week for a maximum of 25 mg/wk subcutaneously, is reported as 50%-80% by retrospective cohort studies in children who failed to respond or were intolerant to thiopurines[3]. MTX is a first-line treatment option in patients who have concomitant inflammatory arthritis and it represents a valuable alternative to maintenance treatment with anti-TNF[3,29]. Adverse events associated with MTX include flu-like symptoms, transaminitis and, less frequently, myelosuppression, which may require dosage adjustment or drug withdrawal[3]. Nausea and vomiting have been reported in 11%-24% of patients, the majority of whom respond to antiemetic medication. Significant hepatocellular liver disease is rare. Contraception is essential[3].

Systematic reviews performed by MacDonald JW and by Patel *et al*[29] to assess the efficacy and safety of MTX for the treatment of active refractory CD in adults, show that intramuscular MTX is effective in inducing remission and acts as a steroid sparing agent allowing complete withdrawal from steroids[28,30,31]. Lower dose oral MTX does not appear to provide any significant benefit in respect to placebo or active comparator[28,31-33]. Though limited by the small size of the studies analysed, this review could not identify any additional benefit from combining MTX and IFX over IFX monotherapy[28,31].

BIOLOGICS AND BIOSIMILARS

TNF is produced by macrophages, adipocytes, fibroblasts and T cells and acts as a pleiotropic, pro-inflammatory cytokine by triggering a cascade of events that lead to tissue damage[5,7].

IFX, ADA and other anti-TNF agents act by suppressing downstream pathways mediated by TNF including angiogenesis, increase in T-helper cell 1 (Th1) cytokine production (interleukin-12 and interferon-γ), death of intestinal epithelial cells and T-cell resistance to apoptosis[5].

Anti-TNF medications are able to achieve and maintain MH, with growing evidence of a change in the natural history of the disease. Importantly, as opposed to a few years ago when clinical response and remission were the main goals in treating patients with IBD, MH has recently been emphasized as a stronger therapeutic goal, as it predicts sustained clinical remission. Therefore, a new concept of "deep remission" has been coined, including MH alongside clinical and/or biochemical remission[18]. Also thiopurines and EEN with polymeric diet have been previously reported to achieve MH, albeit less rapidly and to a lower degree than biologics[18]. According to the studies performed on adult populations, scheduled maintenance therapy with IFX maintains MH in up to 68% of patients, and the subgroup of patients achieving MH show decreased rate of surgeries and hospitalizations[18].

IFX

IFX is a chimeric monoclonal antibody and was the first biologic approved for the treatment of moderate to severe paediatric CD[34]. The advent of biological therapies has drastically modified the treatment strategies and disease course of IBD in children and the role of IFX and ADA in the management of paediatric IBD was recently updated in the Consensus guidelines of ECCO/ESPGHAN[6]. In CD, anti-TNF therapies are currently well established in moderate to severe disease with lack of response to conventional therapy including corticosteroids and immunomodulators, or with contraindications or intolerance to it[34]. Anti-TNF agents are also used as a primary induction option for children with active perianal fistulising disease, in combination with targeted surgical intervention, as well as in children at risk of poor outcomes (top-down treatment)[3,20]. One year response and remission rates for IFX in luminal disease are reported as up to 90% and 55%-60% respectively[35,36]. Repeated administration of IFX can lead to immunogenicity in some patients, with possible loss of efficacy and delayed-type hypersensitivity[34]. A low proportion of children with CD (10%-25%) are primary anti-TNF non-responders, *i.e.*, they fail to respond after the 6 wk induction course. More commonly, however, the formation of antibody against the drug over time can result in secondary loss of response. Concomitant treatment with either thiopurines or MTX has been shown to contain this process[3]. A key step in managing IFX therapy over the more recent years is the increasing availability of therapeutic drug monitoring (TDM), that has improved response rates and has become a most effective tool for the management of secondary failure to IFX[37].

Increasing evidence has shown that trough IFX concentrations < 3 mg/mL during maintenance therapy are associated with treatment failure. Also, children with lower albumin levels have higher IFX clearance and lower drug exposures[38]. Therefore, standard IFX maintenance dosing of 5 mg/kg every 8 wk may not be adequate for all children with CD to achieve sufficient concentration level and thus minimise loss of response (Table 1). Unfortunately, patients who don't respond to one anti-TNF are more likely to also fail a second agent[39]. Researchers hypothesized that a combination therapy of an anti-TNF antibody and another immunomodulator (*i.e.*, thiopurines or MTX) will increase the efficacy and reduce the risk of loss of response[40].

There are several adult trials showing higher treatment efficacy (especially for induction of remission) in patients receiving combination therapy. In particular, concomitant immunomodulators increase IFX levels and reduce immunogenicity[40]. On the other hand, combination therapy in adults exposes patients to the

individual toxicity of both drugs and also seems to increase the risk of malignancy[40]. In paediatric IBD, the safety of combined treatment has been questioned after several cases of hepatosplenic T-cell lymphoma (HSTCL) in young patients with IBD so treated were reported.

Nevertheless, a review by Cozijnsen *et al*[40] points out that almost all studies in paediatric patients with IBD have failed to prove increased benefit from combination therapy compared to monotherapy. Given the controversial aspects above, the Authors suggest to target the use of combination therapy to children with a high risk of serious disease-related complications, such as growth delay, stricturing or fistulising CD phenotype, or panenteric CD[40].

In order to assess the risk of malignancies associated to immunosuppressive treatments in IBD, a retrospective multinational survey of malignancy and mortality in paediatric IBD was conducted over a 6 years period (2006-2011) by the Porto Pediatric IBD working group of ESPGHAN[41]. The most common malignancies identified were hematopoietic tumors (n = 11), of which 3 were HSTCL and 3 Ebstein-Barr virus-associated lymphomas[41]. These 6 patients had all been treated with thiopurines until diagnosis of cancer and only 1 patient had also received 3 IFX infusions, 5 years before the diagnosis of cancer[41]. All 3 patients who developed HSTCL were males and had exposure to thiopurines ranging from 32 to 108 mo; none of them received a biologic[41].

ADA

ADA is a fully human IgG1κ monoclonal antibody which is similar to IFX in the mode of action, but it differs from it as the mouse-derived sequence is removed, in order to reduce the immunogenic reactions induced by chimeric antibodies[23,34]. ADA is therefore to consider as an alternative treatment option in patients who have lost response or are intolerant to IFX, or in some primary non-responders to IFX[34].

ADA was recently approved in Europe and the United States for the treatment of paediatric patients with CD, based on the results from the IMAgINE-1 study[7,42,43].

IMAgINE-1 study is a phase-3, randomized, double-blind study conducted by Sharma *et al*[7] aimed to analyse the PK of ADA in a paediatric CD population (n = 192), and to evaluate the effect of various factors, including demographics (body weight, sex), laboratory measurements (CRP, albumin), previous IFX use, concomitant immunomodulators, and baseline PCDAI, on ADA PK in paediatric patients with moderate-to-severe CD (Table 1). Furthermore, the relationship between serum ADA concentration and remission/response was explored[24].

Trough serum ADA (measured at 5 time points from baseline up to 52 wk) and serial anti-ADA antibody measurements were performed. The study confirmed a strong positive association between serum ADA concentration and clinical remission/response to treatment[24]. Higher body weight, baseline CRP, and lower baseline albumin levels were associated with greater clearance of ADA (lower trough levels)[7]. Previous treatment with other anti-TNF therapy, presence of antibodies to previous IFX therapy, and absence of concomitant use of immunomodulators were also associated with increased clearance of ADA[7]. According to the published experiences from tertiary centres, in anti-TNF antibody naiive children the 1 year remission rate for ADA is 45%[42], and its efficacy has been documented in nearly two-thirds of patients who failed IFX[33]. A retrospective study was conducted by Fumery *et al*[33] who used the population-based EPIMAD registry to evaluate the effectiveness and safety of ADA in children with CD experiencing IFX failure (Table 1).

Twenty-seven children with CD who received ADA before 18 years because of IFX failure or intolerance were included. Clinical response measured by the physician global assessment score after a median follow-up of 16 (8-26) mo was observed in 19 patients (70%). Cumulative probability of failure to ADA treatment at 6 mo and 1 year was 38% and 55%, respectively. Overall, the results from this population-based cohort of paediatric-onset CD with IFX failure show that treatment with ADA was safe and effective in two-thirds of patients. More specifically, ADA was effective in 100% of children intolerant to IFX, 68% of children with secondary failure to IFX, and only 25% of children with primary failure to IFX[33]. A systematic review on the same topic was performed by Dziechciarz *et al*[34]. Who assessed the published evidence on the efficacy and safety of ADA for CD in children (Table 1).

Randomised controlled trials and observational studies (cohort studies, case series of more than 5 patients) were included. A total of 14 studies (1 randomised controlled trial, 13 case series), altogether including 664 patients (age range 1.9-21 years) were available for analysis. The pooled remission rates were: 30% at 4 wk, 42% at 6 mo and 44% at 12 mo. Of the total patients, 6% were primary non-responders and 12% had severe adverse events reported. However, studies differed with respect to patients' characteristics, including percentage of IFX-naïve patients, disease duration, disease localisation, ADA doses, treatment duration, and follow-up period[34].

The Authors' conclusion, though limited by the low-quality evidence based mainly on case series, is that approximately half of the children with CD on ADA therapy achieve remission during the first year of treatment with reasonable safety profile[34].

Vedolizumab

Vedolizumab is a recent biologic treatment, approved for adult patients with IBD in 2014. It is an anti-integrin therapy that blocks the a4b7 integrin receptor molecule

present on the surface of lymphocytes, and thus inhibits the migration of intestinal T-lymphocytes into the tissue[39,44,45]. As this mechanism of action is restricted to the gastrointestinal tract, the risk of systemic immunosuppression (*i.e.*, infections and malignancies) seen with other IBD therapies is mitigated[39].

Its predecessor, natalizumab, acts with a non-specific binding to the a4 chain which causes interference with the lymphocyte trafficking in the central nervous system and led to the concern for reactivation of John Cunningham (JC) virus and subsequent development of progressive multifocal leukoencephalopathy (PML)[45]. Vedolizumab, being gut specific, does not interfere with immune surveillance in the central nervous system, therefore there is no risk of PML as assessed by the placebo-controlled GEMINI studies[44]. Anti-adhesion therapy appears to have a favourable safety profile, though the experience in children is still extremely limited[17]. Due to its mechanism of action of targeting gut-specific T-cell interactions, it is thought that Vedolizumab may not sufficiently treat the extra-intestinal manifestations of IBD. A dual therapy with therapeutic doses of immune-suppressants may therefore be needed to treat extra-intestinal manifestations[39]. So far, Vedolizumab has been particularly effective amongst UC patients, with a clinical response rate of 50% during induction[39]. Whilst the use of Vedolizumab has been approved for the treatment of CD and UC in adults, there is increasing off-label use in paediatric IBD[44].

Singh *et al*[44] conducted a retrospective review to describe the experience with Vedolizumab in 52 children with IBD (58% CD and 42% UC) at 3 tertiary IBD centres and to examine predictors of remission. Ninety percent of the patients had failed at least one anti-TNF agent. Week 14 remission rates for UC and CD were 76% and 42%, respectively ($P < 0.05$). At week 4, eighty percent of anti-TNF-naive patients were in remission. At week 22, anti-TNF-naive patients had higher remission rates than those previously exposed to anti-TNF (100% *vs* 45%, $P = 0.04$). There were no safety concerns.

These results support Vedolizumab as an effective and safe treatment in children with IBD, with UC patients experiencing earlier and higher rates of remission than children with CD. Also, the data reviewed shows that anti-TNF-naive patients had higher remission rates compared to those with previous anti-TNF exposure[44].

Conrad *et al*[39] conducted an observational single-centre prospective cohort study aimed to determine the impact of Vedolizumab on clinical response and on achieving steroid-free remission over 22 wk of therapy. They recruited 21 children with refractory IBD (16 with CD), who had previously failed anti-TNF therapy.

Clinical response was observed in 31.6% and in 57.9% by week 6 and by week 22, respectively. Steroid-free remission was seen in 1 patient at week 6 and in 4 (20%) at week 22. No infusion reactions were observed. Vedolizumab was discontinued in 2 patients because of severe colitis, requiring surgical intervention[39]. These results, though limited by the small sample size, describe a number of children with severe disease who achieve clinical response in the first 6 wk and a further increase in remission rate by week 22[39]. Overall, the data currently available on Vedolizumab from adult and paediatric studies suggests its use as an option to achieve clinical improvement in the most severe paediatric IBD patients (both CD and UC).

Biosimilars

Biosimilars are defined as biological agents that are highly similar to another reference drug already authorized for use[37,46]. This definition also implies that the quality, safety and efficacy of the biosimilar should not be affected by any molecular and/or structural dissimilarities or any potential differences in the underlying mechanisms[37].

Despite keeping the same aminoacid sequence as their reference drug, biosimilars end up being a different final product due to manufacturing process (including cell line, growth condition and purification processes), storage and transport, and subsequent various post-translational modifications (*e.g.*, glycosylation, phosphorylation, sulfation)[37]. Therefore, some uncertainty still exists regarding the exact drug efficacy, immunogenicity and pharmacology of biosimilars in IBD.

In 2013, the EMA authorized two IFX biosimilars, based on two randomized trials on CT-P13 (clinical development name for the biosimilar of Remicade): Remsima (Celltrion Inc., Incheon, South Korea) and Inflectra (Celltrion Inc., Hospira UK Ltd). Because both trials showed equivalent efficacy, tolerance and safety, EMA extended the approval to all indications for which the reference product is labelled, including both adult and paediatric CD and UC[37,46]. At present, a number of IFX biosimilars are licensed for treatment of CD and UC also in the paediatric population.Cost containment remains one of the predominant reasons for development of biosimilars, with a reduction in costs of anti-TNF therapy for IBD by up to 70%[37].

Sieczkowska *et al*[46] conducted a prospective study on 32 children diagnosed with CD and 7 children with UC at 3 academic hospitals in Poland; these patients were switched from IFX originator to its biosimilar (Remsima) (Table 1). Analysis of biosimilar efficacy revealed rates of clinical remission of 88 and 57% for CD and UC patients, respectively, so, in conclusion, switching from IFX originator to its biosimilar was a safe option in this cohort and after the switch, the biosimilar was just as effective as the originator[46].

To date, preliminary results on CT-P13 in IBD are only available from small post-marketing registries and case series with a relatively short-term follow-up period. Although this data suggests comparable efficacy and safety to IFX, more robust post-marketing

studies and pharmacovigilance are warranted to evaluate the bioequivalence of CT-P13 in the coming years[37]. In Europe, at present, in order to switch patients with IBD from IFX originator to its biosimilar, the supervision of the treating physician and the patient's consent are both required[46].

THALIDOMIDE

Thalidomide is an immunosuppressant drug used rarely in the treatment of refractory CD and UC[47]. Its mechanism of action includes several pathways such as inhibition of TNF, IFN-γ and IL-12, stimulation of IL-4 and IL-5 production and, more broadly, a shift in the pattern of lymphocyte cytokine from a Th1 (IFN-γ, IL-12) to Th2 (IL-4, IL-5) type[5]. Thalidomide also interferes with integrin expression, decreases circulating helper T-cells and inhibits angiogenesis[5].

After its suspension due to major teratogenic effects, thalidomide has been more recently re-introduced under FDA approval as an effective treatment for multiple myeloma and severe erythema nodosum leprosum. It is also extensively used off-label for immune-mediated and neoplastic conditions like discoid lupus erythematosus, erythema nodosum leprosum, Behcet's syndrome, aphthous stomatitis, juvenile idiopathic arthritis, brain tumors, graft-*vs*-host disease and IBD[5,18,48].

Thalidomide is used infrequently in the management of paediatric CD, nevertheless it represents a helpful option in treating children who lose response to one or more conventional agents such as thiopurines, MTX, and anti-TNF[48].

Thalidomide (administered from 50 to 400 mg/d in adults and 1.5 to 2.5 mg/kg per day in children[47]) has been shown to induce clinical remission and possibly MH[48]. A systematic review by Yang *et al*[5] selected twelve studies (2 RCTs and 10 case series) where thalidomide was used to induce remission in 248 patients with IBD of all age groups (10 with UC, 238 with CD), 92 of whom were children. Remission rate was 49% and 25%, in adult luminal and perianal CD respectively. In adults with UC, 50% achieved remission and 10% had a partial response. One case series reported 21 patients (17 CD, 4 UC) who maintained remission for 6 mo[5]. Amongst the adverse effects associated with thalidomide and reported in this review, the most common was sedation, in 32.3% of all patients, followed by peripheral neuropathy in 19.8% which was also the main cause of discontinuation[5]. Amongst the studies in this review, one high quality RCT showed that thalidomide is effective in inducing remission in paediatric CD[49]. Based on the evidence reviewed and given the limited data available, the Authors support the use of Thalidomide in the most severe cases of IBD for induction or maintenance of remission[5].

Lazzerini *et al*[49,50] conducted randomized placebo-controlled trials of thalidomide in both paediatric CD and UC (Table 1). In their CD trial, they randomized 52 children to receive thalidomide or placebo; almost all patients had not responded to thiopurines, and about 35% had also not responded to biologics. The majority of children had luminal disease and few had perianal disease. Although no significant response was noted at 4 wk, by 8 wk 46% of children treated with thalidomide had a reduction in their PCDAI greater than 75%, compared with 12% amongst patients treated with placebo[49].

Their UC trial enrolled 26 children with active UC who were randomized to receive thalidomide or placebo. All patients had active disease despite thiopurines, and about 35% had received previous IFX treatment. The UC children treated with thalidomide achieved higher remission rates at 8 wk (83% *vs* 19% for placebo) than those in the CD trial[50].

Both trials showed that thalidomide does not work quickly, as there were no significant differences between placebo and thalidomide at 4 wk, whereas major differences were seen by 8 wk[49,50]. These trials also support the use of Thalidomide as a maintenance agent in cases of refractory CD or UC, for example patients with secondary loss of response to biologics[49,50]. Another more recent systematic review conducted by Bramuzzo *et al*[47] analysed 722 papers, including two randomized controlled trials and 29 uncontrolled studies for a total of 489 patients, 135 (28.4%) of whom were children.

Overall, thalidomide appeared to be a promising therapy for IBD: induction of clinical remission was achieved in 51.4% of cases, and in 69.3% a clinical response was observed in the first months of treatment. In almost 50% of the cases in which endoscopy was performed, complete MH was observed and a further 15% of patients showed an improvement in their endoscopy score[47]. IBD remission was maintained in 72.2% after 12 mo and in 54.5% after 2 years[47].

Adverse events leading to drug suspension had a cumulative incidence of 19.7/1000 patients-month, with neurological symptoms being the main cause[48]. This review highlights that thalidomide is an effective alternative in patients who fail biologic treatment, which is most likely due to the different mechanism of action of Thalidomide compared to anti-TNF agents[47]. Careful precautions must be taken to avoid its use in pregnant women. No case of teratogenicity has been observed in 124000 patients enrolled in the thalidomide distribution risk management program for more than 6 years[49,50]. Other reported side effects of thalidomide include peripheral neuropathy (clinical or subclinical, primarily with axonal damage) followed by sedation, constipation, mood disturbances, skin rash, pedal oedema, neutropenia and deep vein thrombosis[5,48].

STUDIES ON NEW TREATMENTS

New biologics and other agents are being tested

in phase Ⅱ and Ⅲ trials on adult patients with IBD and are therefore on the horizon within the field of paediatric gastroenterology as well. Examples are the IL-23 inhibitor risankizumab and the IL 12- IL 23 inhibitor ustekinumab. Drug therapies that interfere selectively with lymphocyte trafficking are also emerging treatment options for UC[11,51]. Etrolizumab is a humanized monoclonal antibody against the b7 integrin subunit that acts by reducing the homing of leukocytes to the gut mucosa and the retention of lymphocytes in the epithelium[11]. It has recently been tested in a phase 2 study showing efficacy in patients with moderate to severe UC, compared to placebo[11]. Levels of granzyme A (GZMA) and integrin aE (ITGAE) mRNAs in colon tissues can identify patients with UC who are most likely to benefit from etrolizumab[11] (Table 1).

Another agent recently developed is Ozanimod (RPC1063), an oral agonist of the sphingosine-1-phosphate receptor subtypes 1 and 5 that induces sequestration of peripheral lymphocytes, and as a consequence a decrease in the number of activated lymphocytes circulating to the gastrointestinal tract[51]. Its agonists induce internalization and degradation of the S1P1 receptor, which makes B and T lymphocytes incapable of migrating from secondary lymphoid organs, with subsequent reduction in circulating lymphocytes[51]. Being an oral formulation, Ozanimod represents an alternative to infusions of monoclonal antibodies for the treatment of UC, with no risk of sensitization and formation of antidrug antibodies. On the other hand, this product can be less selective than monoclonal antibodies, which may expose to adverse effects[51].

Sandborn *et al*[51] performed a double-blind, placebo-controlled phase 2 trial of Ozanimod in 197 adults with moderate-to-severe UC. Clinical remission at 8 wk was observed in 16% of the patients who received 1 mg of Ozanimod and in 14% of those who received 0.5 mg, as compared with 6% of those who received placebo (P = 0.048, P = 0.14, respectively) (Table 1).

At week 32, the rate of clinical remission was 21% in the group that received 1 mg of Ozanimod, 26% in the group that received 0.5 mg of Ozanimod, and 6% in the group that received placebo; the rate of clinical response was 51%, 35%, and 20%, respectively[51].

At week 8, absolute lymphocyte counts dropped by 49% from baseline in the group that received 1 mg of Ozanimod and by 32% from baseline in the group that received 0.5 mg[51]. The most common adverse events overall were anemia and headache[52].

From this preliminary trial, the Authors conclude that Ozanimod at a daily dose of 1 mg is more effective than placebo in inducing clinical remission of UC. However, complete assessment of clinical efficacy and safety could not be achieved by this trial due to limitations in size and duration[51]. Another monoclonal antibody recently investigated is the anti-natural killer group 2 member D (NKG2D), which acts by antagonising the human immunoglobulin G4 that binds to NKG2D receptors.

The interaction between intestinal epithelial cells and T-cells in the gut mucosa has a key role in T-cell regulation. NKG2D receptors are expressed by T cells and innate lymphoid cells, and exhibit pro-inflammatory properties. Upregulated NKG2D ligands on epithelial cells in the inflamed tissue of patients with IBD may activate the proliferation of several subsets of effector T-cells, leading to increased production of pro-inflammatory cytokines and enhanced cytotoxicity[52]. Previous trials have demonstrated an increase in effector T cells as well as in the expression of NK receptors on T cells in patients with CD[52]. Also, neutralising anti-NKG2D antibodies have been shown to reduce inflammatory-induced colitis in murine models[52].

Allez *et al*[52] performed a randomised, double-blind, parallel group trial of a single subcutaneous dose of 2 mg/kg anti-NKG2D or placebo in 78 adult patients with active CD (Table 1).

Change in CDAI from baseline to week 4 was the primary end-point, and was not found to differ significantly between anti-NKG2D and placebo; however, a significant difference was observed by week 12 ($P \leqslant 0.10$)[52]. The group of patients who hadn't previously failed biologics and were treated with anti-NKG2D (n = 28) showed the most significant improvement from week 1 onward. Most adverse events were mild (49%) or moderate (43%). No antidrug antibodies were detected[52].

Based on the results of this trials, the Authors conclude that a single s.c. dose of 2 mg/kg anti-NKG2D did not reduce disease activity at week 4, but it showed significant response rate at week 12. Therefore, there is evidence to consider further investment in anti-NKG2D in IBD[52].

DISCUSSION

Though major progress has been achieved in treating IBD patients of all ages, there are still significant limitations in what is currently available to manage this condition. The advent of new biologics and other medications targeting pathways previously unexplored (*e.g.*, leukocyte trafficking through the gut mucosa) has provided clinicians with more hope for patients who fail to respond to current treatments. Nevertheless, whilst the causative mechanisms underlying IBD are not yet fully understood, treatment options can only target downstream components of this inflammatory chain.

IBD, and CD in particular, are difficult to categorise as distinct disease entities, as the spectrum of its clinical phenotype is very broad and variable, with mild disease responding to standard treatments, and severe disease often developing to structuring or penetrating

Table 2 Summary and take home messages

1. The pathogenesis of IBD is not completely understood yet, and all therapies currently available are aimed at downstream steps of the complex inflammatory process. Specific targets when treating children with IBD are achieving satisfactory growth and nutritional status.
2. Paediatric pharmacometric approaches are increasingly applied to drugs already in use but that remain unlicensed and off-label in children due to missing information on age appropriate dosing, efficacy and safety.
3. Corticosteroids can be used to induce remission in CD (when exclusive enteral nutrition is not possible) and are first-line therapy for induction of remission in UC, particularly in case of non-response to 5-ASA or with severe presentation.
4. One of the targets of current research is to customise therapy to a patient's predictive biomarker profile in order to personalise treatments and to maximise response.
5. 5-ASA are used in induction and maintenance of remission in UC. They are not recommended in CD except from post-operative maintenance of remission.
6. Thiopurines include AZA and 6-MP and are steroid sparing agents. They are effective in maintaining disease remission in patients with CD and UC as well as post-surgical remission in CD. The use of TPMT activity and 6-TGN level measurements helps avoiding nearly a quarter of episodes of myelosuppression as well as to monitor non-compliance, under-dosing, drug-resistance or refractory state.
7. An increase in mean 6-TGN blood level has been reported in patients on 6-MP or AZA co-treated with 5-ASA, with a higher rate of myelotoxicity in respect to patients treated with the thiopurine alone.
8. MTX is effective in 50%-80% of the children who fail to respond or are intolerant to thiopurines. It is particularly suitable to patients who have coexistent inflammatory arthritis.
9. In CD, anti-TNF agents are used to treat moderate to severe disease with inadequate response, or contraindication to, or intolerance to, conventional therapy including corticosteroids and immunomodulators. They are also indicated in children with active perianal fistulising disease. Therapeutic drug monitoring of IFX has improved response rates and is increasingly used in clinical practice as a tool for the management of secondary failure to IFX.
10. ADA is a fully human IgG1k monoclonal antibody, which represents a treatment option in patients who have lost response or are intolerant to IFX. ADA achieves remission rates of 45% at 1 yr in anti-TNF naïve children and is effective in nearly 2/3 of patients with IFX failure.
11. Vedolizumab is an anti-integrin therapy which inhibits the migration of intestinal T lymphocytes. The mechanism of action of Vedolizumab is restricted to the GI tract, mitigating the risks of systemic immunosuppression such as infections and malignancies. The clinical response rate for induction with vedolizumab is 50% in UC patients. Vedolizumab is approved for treatment of CD and UC in adults and there is increasing off-label use in children.
12. Thalidomide is an immunosuppressant drug used infrequently off-label in the treatment of refractory CD and UC. Induction of remission is achieved in aroud 50% cases and clinical response in 70%.
13. Drug therapies that interfere selectively with lymphocyte trafficking (*e.g.*, etrolizumab, ozanimod) are emerging treatment options for UC.

UC: Ulcerative colitis; IBD: Inflammatory bowel disease; ADA: Adalimumab; IFX: Infliximab; TPMT: Thiopurine methyl transferase; 5-ASA: Aminosalicylates; CD: Crohn's disease; 6-MP: 6-mercaptopurine; AZA: Azathioprine; MTX: Methotrexate; GI: Gastrointestinal.

phenotypes that require surgery, despite the use of multiple treatment escalations[53]. Once significant chronic bowel damage occurs in IBD, the chances of recovery with medical treatments alone are limited. Therefore, the ideal treatment should be offered before complications develop[53]. Step-up and top-down approaches have been debated for different risk groups of children with IBD. The management of paediatric IBD has evolved significantly over recent years with evidence-based guidelines in place to provide uniform and solid guidance in clinical practice. Nevertheless, a long-term response is only observed in less than half of the patients with CD[3,52]. Although new biologics are continuously being developed, primarily monoclonal antibodies targeting the trafficking of immune cells, we certainly are in need of more therapies with novel mechanisms of action[52]. More significant advances have been achieved in monitoring drug administration and the response to medications, in particular the clinical availability of AZA metabolites, IFX and ADA trough levels and anti-IFX antibody measurements in clinical practice (*i.e.*, TDM). This has allowed a major step forward in monitoring and targeting patients' treatments with a more personalised approach.

Despite routine use of solid clinical scores (*e.g.*, PCDAI, PUCAI) and biochemical parameters (blood-based and stool-based), we are currently still depending on invasive reassessments (*i.e.*, endoscopic procedures) for an adequate monitoring of the disease course. One next goal on the horizon is therefore the development of reliable biomarkers to be used for prediction of prognostic outcomes in IBD. The ability to stratify individual patients' risk would allow clinicians to personalise treatment from disease presentation, tailoring more potent drugs (possibly in combination) to patients with a high risk of severe disease, using more standard options for patients who are destined for a milder disease course[53]. This individualised approach requires reliable prognostic biomarkers and hence major efforts are being made in the development of such markers. Risk stratification models would allow clinicians to treat patients effectively before complications arise, and to optimise majorly the management of patients with IBD as improve the cost effectiveness of their care[53].

CONCLUSION

Our narrative review summarises some of the recent advances in treating children with IBD (Table 2). Whilst the mechanisms underlying this condition are yet to be fully understood, both old and new generation treatments can still only target known pathways of what is a very complex pathogenesis. A significant proportion of children with IBD does not respond to currently available treatments, either at diagnosis

or during disease course, therefore new treatment options are urgently needed. Major advances have been achieved in monitoring the individual metabolism, toxicity and response to treatments in IBD. Amongst the priorities in current research is the development of reliable prognostic biomarkers, an essential step towards the personalised treatment of patients with IBD.

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P- Reviewer: Matowicka-Karna J, Tambuwala M, Zhang MZ
S- Editor: Qi Y **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5486-5498

DOI: 10.3748/wjg.v23.i30.5486

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

MINIREVIEWS

Influence of gut microbiota on neuropsychiatric disorders

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Author contributions: Cenit MC wrote the first draft of the manuscript; Sanz Y and Codoñer-Franch P have contributed substantially to the writing and revising of the manuscript; Codoñer-Franch P designed the aim of the editorial; all the authors have made substantial intellectual contributions, take responsibility to the paper and give final approval of the version to be submitted.

Supported by Spanish Ministry of Economy and Competitiveness (MINECO), No. AGL2014-52101-P; A Sara Borrell postdoctoral fellowship from the Instituto de Salud Carlos III at the Spanish Ministry of Health (Spain), No. CD14/00237 (Cenit MC).

Conflict-of-interest statement: All the authors declare no conflict of interest related to this publication.

Manuscript source: Invited manuscript

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Received: February 8, 2017
Peer-review started: February 9, 2017
First decision: April 17, 2017
Revised: May 8, 2017
Accepted: July 4, 2017
Article in press: July 4, 2017
Published online: August 14, 2017

Abstract

The last decade has witnessed a growing appreciation of the fundamental role played by an early assembly of a diverse and balanced gut microbiota and its subsequent maintenance for future health of the host. Gut microbiota is currently viewed as a key regulator of a fluent bidirectional dialogue between the gut and the brain (gut-brain axis). A number of preclinical studies have suggested that the microbiota and its genome (microbiome) may play a key role in neurodevelopmental and neurodegenerative disorders. Furthermore, alterations in the gut microbiota composition in humans have also been linked to a variety of neuropsychiatric conditions, including depression, autism and Parkinson's disease. However, it is not yet clear whether these changes in the microbiome are causally related to such diseases or are secondary effects thereof. In this respect, recent studies in animals have indicated that gut microbiota transplantation can transfer a behavioral phenotype, suggesting that the gut microbiota may be a modifiable factor modulating the development or pathogenesis of neuropsychiatric conditions. Further studies are warranted to establish whether or not the findings of preclinical animal experiments can be generalized to humans. Moreover, although different communication routes between the microbiota and brain have been identified, further studies must elucidate all the underlying mechanisms involved. Such research is expected to contribute to the design of strategies to modulate the gut microbiota and its functions with a view to improving mental health, and thus provide opportunities to improve the management of psychiatric diseases. Here, we review

the evidence supporting a role of the gut microbiota in neuropsychiatric disorders and the state of the art regarding the mechanisms underlying its contribution to mental illness and health. We also consider the stages of life where the gut microbiota is more susceptible to the effects of environmental stressors, and the possible microbiota-targeted intervention strategies that could improve health status and prevent psychiatric disorders in the near future.

Key words: Microbiota; Microbiome; Dysbiosis; Brain-gut axis; Mental health; Psychiatric conditions

Core tip: The gut microbiota has been revealed as an additional regulator of the gut-brain axis, which may be involved in many neurodevelopmental and neurodegenerative disorders. The modulation of this axis is currently being explored, targeting the gut microbiota in endeavors to improve mental health, especially in early and late life. So far, most of our knowledge is based on animal trials, in which interventions with pro and prebiotics have shown promising results regarding efficacy. Nevertheless, we require further understanding of how the microbiota regulates gut-brain communication and function in order to establish the rationale behind microbiota-based interventions.

Cenit MC, Sanz Y, Codoñer-Franch P. Influence of gut microbiota on neuropsychiatric disorders. *World J Gastroenterol* 2017; 23(30): 5486-5498 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5486.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5486

INTRODUCTION

Research into the influence of human genetics on numerous conditions, including neuropsychiatric disorders, has been underway for many years; however, the etiology of most of these conditions has yet to be unraveled. As in other multifactorial conditions, there is considerable discordance in the development of neuropsychiatric disorders between monozygotic twins, indicating that non-genetic factors are also involved[1,2]. Nowadays we know that both the human genome and the genome of the gut microbiota (microbiome) are essential for maintaining health, since the latter also plays a crucial role in regulating important aspects of host physiology, including brain development and function[3,4]. Indeed, different studies have reported that gut microbiota is able to shape brain physiology and thus behavior through the gut-microbiota-brain axis and have suggested gut microbiota as a key trigger factor in the development of many neuropsychiatric conditions[5]. Most of the neuropsychiatric disorders are considered as multifactorial disorders prompted by certain environmental factors in genetically susceptible individuals. However, there is a need of further work to elucidate the exact complex gene-environment interactions and gut microbiota alterations that precede the onset of the different neuropsychiatric diseases and their manifestations in order to decipher the etiology of the neuropsychiatric disorders.

It is noteworthy that the microbiota is more "medically" accessible and modifiable than the human genome. This fact provides a promising opportunity for preventing or treating neuropsychiatric conditions[6]. In this respect, studies in animal models, where the intestinal microbiota can easily be manipulated, have shed light on how the microbiota may be involved in the development of certain mental diseases. In fact, different communication routes between the microbiota and brain have already been identified[7] although further studies are required to elucidate the underlying mechanisms. Various studies also indicate that the activity of the gut microbiota can modify the host epigenome impacting on gene expression[8]; furthermore, epigenetic mechanisms are involved in neurogenesis, neuronal plasticity, learning and memory, and in disorders such as depression, addiction, schizophrenia and cognitive dysfunction[3]. Consequently, it has been suggested that gut microbiota may be involved in the pathogenesis and risk of developing neuropsychiatric disorders through epigenetic modifications, which are highly dynamic and reversible[3]. Thus, it is tempting to speculate that modulating the microbiota and its metabolic products will enable us to modulate the epigenome and, thereby, prevent or treat mental illness. In this respect, metabolites produced by the microbiota from fiber fermentation are known to inhibit histone deacetylases (HDACs) and reduce inflammation through epigenetic modifications[9].

Currently it is well accepted that our gut microbiota is critical for brain processes such as myelination, neurogenesis and microglial activation and can effectively modulate behavior and influence psychological processes such as mood and cognition[10]. Indeed, very recently gut microbiota have been shown essential for the maintenance of microglia in a healthy functional state[11], which is necessary for the prevention of neurodevelopmental and neurodegenerative disorders[12].

The early assembly of a well-balanced microbiota composition and its subsequent maintenance is considered crucial for human health as perturbations negatively impact health and increase host susceptibility to a wide variety of diseases, including behavioral and neuropsychiatric disorders[3,4]. In this respect, three critical time windows have been proposed including infancy, adolescence and ageing, when the gut microbiota is more vulnerable to external influence[13]. Therefore, strategies aiming to target the

Table 1 Preclinical evidences of the role of gut microbiota on behavior

Germ-free (GF) mice have shown impaired social behavior[39]
GF mice have displayed exaggerated stress response[21] and differences in anxiety-like behavior[22,23]
GF mice have showed crucial changes in multiple neurotransmitters and their receptors in different brain regions[23]
GF animals have exhibited an impaired neurogenesis[25] and structural and functional changes in the amygdala[26]
GF mice have shown prefrontal cortical hypermyelination[27]
Microglial function impaired in GF animals is rescued by the oral treatment with short chain fatty acids[11]
Gut microbiota has been shown to modulate brain-derived neurotrophic factor, oxytocin and vasopressin brain levels[20]
Different probiotic preparations for administration to rats and mice have shown to achieve a reduction in anxiety-like and depressive-like behaviors[6,58]

gut microbiota might have a greater impact at those stages of life, *i.e.,* newborn, adolescence and elderly populations.

Many factors, including human genetics, influence the gut microbiota composition; therefore the microbiota constitutes a highly dynamic ecosystem, with high inter-individual variability[14,15] and this indeed hampers the understanding of the role of gut microbiota in the etiology and progress of neuropsychiatric diseases.

Nowadays, each adult individual is believed to harbor a unique gut microbiota composition, as personal as a fingerprint, and certain early life events may be important contributors to the individual's microbiota, including mode of delivery, type of feeding, medication, stress and infections[15]. The critical gut microbiota developmental period occurs in parallel to growth, maturation and sprouting of neurons in the young brain. In fact, childhood and adolescence represent the most dynamic and vulnerable periods for both gut microbiota composition and neuronal development[16]. Furthermore, although the symbiotic link between the host and the microbiota seems to be established early in life, the gut microbiota composition may still experience changes in adulthood despite its greater resilience to the effect of detrimental environmental factors. Likewise, it is also well recognized that ageing is associated with reduced microbial diversity and that healthy ageing correlates with a diverse microbiota[17]. Furthermore, research shows that as we age there is a decline in microbiota complexity parallel to a decrease in neuronal complexity and, altogether, those changes may lead to an increased risk of neurodegenerative disorders[18]. Nowadays, it is well recognized that the onset of most of the neuropsychiatric disease really often is close to a period where the gut microbiota is more unstable and, therefore, at risk of suffering microbiota alterations. Despite these findings, there is currently a need for longitudinal studies in humans to assess the impact of the gut microbiota dynamics on the maintenance or decline of neurocognitive function and to understand to what extent results in animal models can be generalized to humans[19].

In this review, first we summarize the current evidence for a role of the gut microbiota in brain development and function, and also summarize the state of the art on the different mechanisms involved. Thereafter, we provide an update on research into specific neuropsychiatric disorders. We also refer to the different stages of gut microbiota development and maturation, identifying the periods where the gut microbiota is more unstable and, therefore, at greater risk of suffering microbiota alterations due to exposure to stressors. Lastly, we briefly highlight different microbiome intervention strategies that might be implemented to improve the management of psychiatric diseases.

MICROBIOTA, BRAIN DEVELOPMENT, FUNCTION AND BEHAVIOR

To investigate the role of the gut microbiota in the gut-brain axis, numerous approaches have been taken using animal models, including the study of microbiota deficient animals, known as germ-free (GF) mice, and of animals treated with antibiotics or with specific bacterial species. Such studies have provided new insights into how the microbiota is involved in regulating brain development and function (Table 1)[20,21].

Recently, different studies using GF mice have demonstrated that animals completely lacking microbiota have impaired social behavior, as well as other types of behaviors such as anxiety and stress response[22-24]. Furthermore, it has been observed that certain behaviors induced by the absence of gut microbiota correlate with neurochemical changes in the brain[24]. Said studies have also shown crucial changes in multiple neurotransmitters and their receptors in different brain regions of GF mice. Moreover, the ability to transfer behavioral traits using fecal microbiota transplantation has also been demonstrated, suggesting that some microbiota changes could be rather a cause than a consequence of behavioral alterations[25].

Recent data obtained using GF animals have shown that neurogenesis, a process that plays a critical role in modulating learning and memory, is also regulated by the microbiome[26]. Furthermore, the gut microbiota is reported to modulate structural and functional changes in the amygdala, a critical brain area for social and fear-related behaviors, which are associated with a variety of neuropsychiatric disorders[27].

Another aspect of neurodevelopment shown to be critically regulated by the microbiome is prefrontal cortical myelination[28]. A recent study also showed that depletion of the gut microbiota as of early adolescence

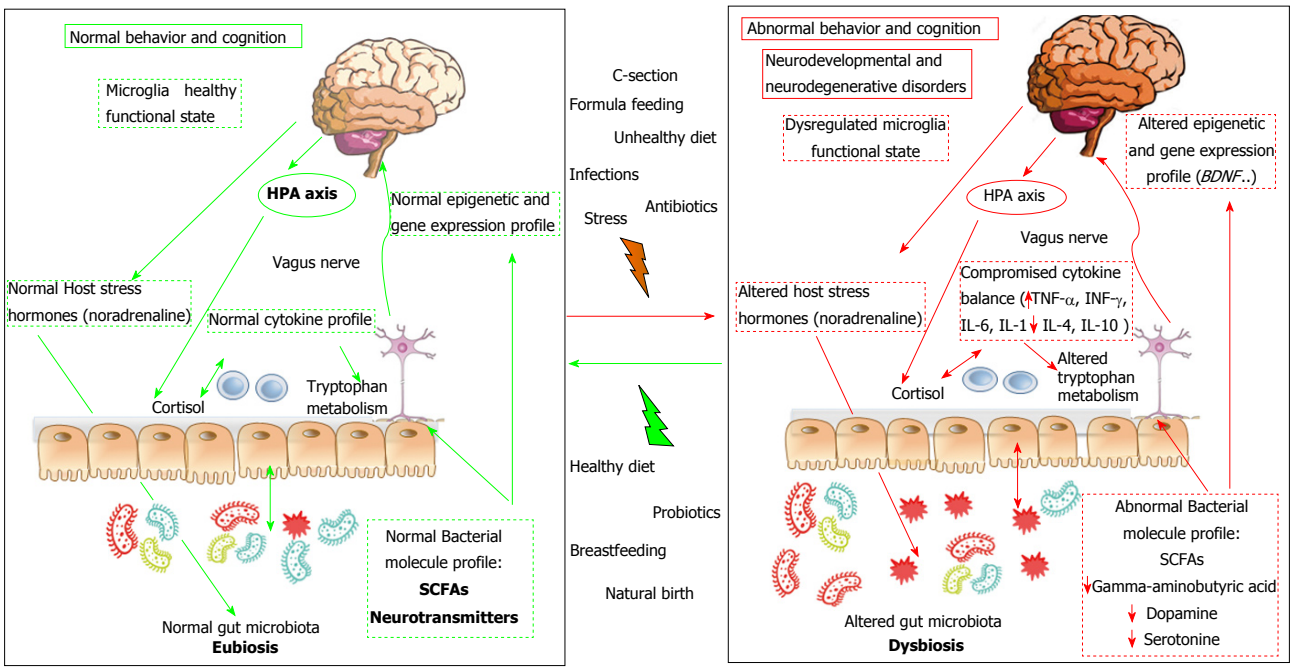

Figure 1 Schematic representation of the mechanisms involved in the relationship between microbiota and brain development and function: Cytokine balance and microglia activation (immune pathway), cortisol (endocrine pathway) and vagus and enteric nervous system (neural pathway). The axis plays an important role in homeostasis and has been linked to several disorders. Altered gut microbiota composition enhances the risk of neurodevelopmental and neurodegenerative disorders possibly from microbiota-derived products such as small chain fatty acids and neurotransmitters. HPA: Hypothalamic-pituitary-adrenal.

in mice alters their behavior and significantly reduces brain-derived neurotrophic factor (BDNF), oxytocin and vasopressin expression in the adult brain[21].

Very recently it has been demonstrated that the maturation and activation of microglia, the macrophages of the brain crucial for maintaining brain tissue homeostasis, are also regulated by the gut microbiota[11]. The same study demonstrated that treatment with microbial-produced short-chain fatty acids (SCFAs) could rescue microglial function impaired in GF animals[11]. In addition, various studies have shown that probiotic administration to healthy rats and mice can alter behavior, achieving a reduction in anxiety-like and depressive-like behaviors and thus highlighting the beneficial effects of probiotics on stress-related behaviors[6]. All these findings indicate that probiotics may have broader therapeutic applications than previously considered, particularly in the area of anxiety and depression[6].

GUT MICROBIOTA MECHANISMS MODULATING BRAIN DEVELOPMENT AND FUNCTION

Recent studies have provided insights into the possible pathways and mechanisms that connect the microbiota to the brain. In fact, recent evidence largely suggests that there are several mechanisms by which microbiota may modulate brain development, function and behavior including immune (cytokines), endocrine (cortisol) and neural (vagus and enteric nervous system) pathways (Figure 1). Likewise, different mechanism have been identified by which also brain can influence the gut microbiota composition[7]. Results of animal studies show that stress and emotions cause the brain to influence the microbial composition of the gut through the release of hormones or neurotransmitters, which influence gut physiology and alter the habitat of the microbiota, resulting in preferential growth of certain communities. Indeed, host stress hormones such as noradrenaline might influence bacterial gene expression or signaling between bacteria, and this might change the microbial composition and activity of the microbiota. In addition, the microbiota has a substantial impact on the metabolomics profile of the host. It is important to highlight that a large array of crucial molecules with neuroactive functions is produced by microbes[7]. Nowadays it is clear that certain bacteria are strain-specifically able to produce different essential neurotransmitters and specific neuromodulators. Indeed, several neurotransmitters such as gamma-aminobutyric acid (GABA), serotonin, catecholamines and acetylcholine are produced by bacteria, some of which are inhabitants of the human gut. Indeed, researchers report that *Lactobacillus* spp. and *Bifidobacterium* spp. produce GABA[29]; *Escherichia* spp., *Bacillus* spp. and *Saccharomyces* spp. produce noradrenalin; *Candida* spp., *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp. produce serotonin; *Bacillus* spp. produce dopamine; and *Lactobacillus* spp. produce acetylcholine[6]. Neurotransmitters secreted from bacteria in the intestinal lumen may induce epithelial cells to release molecules that, in turn, have the ability to modulate neural signaling within the enteric nervous system

and subsequently control brain function and behavior. Various bacterial strains have also been shown to mediate behavioral effects *via* the vagus nerve in some animal studies although vagotomy does not seem to mediate all microbiota-mediated effects on brain function and behavior[30].

Tryptophan is an essential amino acid precursor to many biologically active molecules, including the neurotransmitter serotonin and metabolites of the kynurenine pathway. Only around 5% of systemic tryptophan is metabolized into serotonin and the rest is metabolized along the kynurenine pathway. This depends on the expression of two enzymes, indoleamine 2,3-dioxygenase, which is found in all tissues, and tryptophan 2,3-dioxygenase, which is localized within the liver. The activity of both enzymes is strongly controlled by inflammatory mediators such as cytokines and corticosteroids. The increased activation of these two enzymes could induce serotonin depletion and depressive mood. Furthermore, the downstream metabolites of the kynurenine pathway are neuroactive metabolites, which can also modulate neurotransmission. In addition, the oral ingestion of *Bifidobacterium infantis* led to increased levels of the serotonin precursor, tryptophan, in the plasma of rats, suggesting that this specific strain may be a potential antidepressant. Other studies have also demonstrated the effect of the gut microbiota on the levels of other metabolites related with tryptophan metabolism[31].

Other important molecules, which are produced in the colon by microbial fermentation of dietary fiber, are SCFAs such as butyrate, acetate and propionate. SCFAs are known to have neuroactive properties; for instance, the administration of a high dose of propionate in rats induced a neuroinflammatory response and behavioral alterations related with neurodevelopmental disorders[32]. Propionate is also a common preservative in food products that has been demonstrated to exacerbate autism spectrum disorder symptomatology. Moreover, butyrate decreases depressive-like behavior with parallel changes in histone deacetylation and BDNF expression. SCFAs also regulate the gut immune system and this may have consequences on the central nervous system. As mentioned above, the maturation and activation of microglia is also regulated by the gut microbiota[11] and oral treatment with microbial produced SCFAs can rescue microglial function impaired in GF animals[11]. However, it is still unclear whether SCFAs produced in the gut can cross the blood-brain barrier.

EPIGENETICS, GUT MICROBIOTA AND NEUROLOGICAL CONDITIONS

It is currently well recognized that there are many changes in gene expression not caused by variations in sequence or genotype. Those changes are rather triggered by epigenetic modifications, such as methylation, acetylation or non-coding RNAs (ncRNAs), which modulate chromatin remodeling and the final translation of coding mRNA into proteins. Several types of epigenetic-modifying enzymes together with ncRNAs are involved in epigenetic regulation and have been demonstrated highly sensitive to environmental changes. Therefore, epigenetic modifications associated to many diseases have been recognized as possible pieces missing in the puzzle linking the human genome, environment and phenotype development[33]. Epigenetic mechanisms are currently known to be involved in neurogenesis, neuronal plasticity, learning and memory, and in disorders such as depression, addiction, schizophrenia and cognitive dysfunction[3,34]. Many of the environmental factors apparently playing a crucial role in the etiology of neuropsychiatric disorders might be related with the risk of developing the condition through epigenetic mechanisms.

Changes in histone modifications and DNA methylation have been found at the promoters of genes involved in neuropsychiatric conditions[3]. For instance, chromatin remodeling at the BDNF gene promoter has been associated with neuronal activity and stress and likely affects many more genes involved in brain function and behavior[35]. In fact, there is now a great deal of evidence for the role of epigenetic regulation in shaping brain function and behavior, even though the underlying molecular mechanisms by which epigenetics might be leading to the behavioral and biochemical alterations observed in neuropsychiatric disorders are still not well understood[36].

Gut microbiota has been shown to impact host gene expression by modulating epigenetic processes which are highly dynamic and reversible[33]. Therefore, it is tempting to speculate that modulating the microbiota will enable us to shape our epigenome in the near future. Indeed, the methylation levels of specific genes involved in metabolism and inflammatory responses have been associated with gut microbiota profiles[37]. Furthermore, various studies have described a link between microRNAs, a group of small ncRNAs, and microbiota[38]. Histone deacetylations by HDACs are also critical in epigenomic regulation and are related with condensed chromatin and, consequently, the inhibition of the gene expression. Indeed, SCFAs have the capacity to inhibit HDACs, thus activating the gene expression of previously deacetylated genes[33].

Aging is associated with profound epigenetic changes resulting in alterations in gene expression and also with a wide range of human disorders, including neurodegenerative diseases. Therefore, the reversibility of epigenetic changes that occur as a hallmark of aging offers exciting opportunities to treat age-related diseases[39].

GUT MICROBIOTA AND NEUROPSYCHIATRIC CONDITIONS

So far many evidences derived from multiple studies performed in both animal models and humans have

Table 2 Current evidences linking gut microbiota to neuropsychiatric disorders

Autism
Increase in microbiota diversity is associated with autism[43]
Abundance of Bacteroidetes has found to be linked with severe autistic cases[43]
Increase in short chain fatty acids has been found in fecal samples from autistic children[44]
A specific strain of the species *Lactobacillus reuteri* has shown to modulate oxytocin levels and reverse autism-related behavior[41]
Schizophrenia
Dopamine, the key neurotransmitter associated with schizophrenia pathophysiology, is produced by components of the microbiota[53]
Increased gastrointestinal inflammation is associated with schizophrenia[53]
Intake of antibiotics is associated with the risk of schizophrenia[54]
Attention deficit hyperactivity disorder
The risk of developing ADHD has been suggested to be associated with many perinatal risk factors, including delivery mode, gestational age, type of feeding, maternal health and early life stressors, all of them linked to gut microbiota alterations[56]
Dietary components modulating gut microbiota may influence ADHD development or symptoms[56]
Depression
Increase in gut microbiota alpha diversity is associated with depression[59,63]
Lower numbers of Bifidobacterium and Lactobacillus have been found in individuals with depression[60]
Increases in the genus *Eggerthella, Holdemania, Gelria, Turicibacter, Paraprevotella and Anaerofilm*, and reductions in *Prevotella* and *Dialister* have been found in individuals with depression[61]
A negative correlation between *Faecalibacterium* spp. and severity of depressive symptoms has been reported[61]
Role of diet on depression onset is suggested (Mediterranean diet seems to protect, whereas Western diet seems to be associated with an increased risk)[64]
Different strains of *Lactobacillus rhamnosus, Lactobacillus helveticus, Bifidobacterium longum, Bifidobacterium breve and Bifidobacterium infantis* have been shown to attenuate depression and anxiety-related behavior in rodents[58]
A probiotic combination (*Lactobacillus helveticus R0052 and Bifidobacterium longum R0175*) has proven effective in increasing the subject's resilience to stress in humans[57]
Parkinson's disease
Alterations in bowel function, mainly constipation, often precede the onset of motor symptoms associated with PD[76]
Reduction in the levels of Prevotellaceae has been found in PD patients[80]
Positive correlation between levels of Enterobacteriaceae and the severity of postural instability and gait difficulty was proven in PD patients[80]
Reduction in short chain fatty acids[78] and butyrate-producing bacteria (*Blautia, Coprococcus, Faecalibacterium spp and Roseburia*)[79] were found in fecal samples from PD patients
GF mice overexpressing human α-synuclein (αSyn) display reduced microglia activation, αSyn aggregates and motor deficits (treatment with short chain fatty acids restored all major features of PD in GF mice)[77]
Gut microbiota transfer from PD patients into GF mice overexpressing human α-synuclein (αSyn) enhances physical impairments whereas gut microbiota transfer from healthy human donor does not enhances those deficiencies[77]
Alzheimer's disease
Risk factors for AD such as metabolic syndrome, type 2 diabetes and obesity are associated with gut microbiota alterations[86,87]
Gut microbiota seems to be involved in the accumulation of amyloid plaques according to the results of a study using a mouse model of AD[88]

AD: Alzheimer's disease; PD: Parkinson's disease; ADHD: Attention deficit hyperactivity disorder.

strongly suggested a link between gut microbiota and development and/or manifestation of different neuropsychiatric conditions (Table 2).

Microbiota and impairment of social behavior (autism), schizophrenia and attention deficit hyperactivity disorder

Studies using GF mice have demonstrated that animals completely lacking microbiota exhibit deficiencies in social behavior. In particular, John Cryan's research team examined the behavior of GF mice in the three-chamber test and observed that GF mice spent as much time with the familiar as with the novel mouse in contrast to the behavior of conventionally colonized mice, which spent more time with the novel than the familiar mouse[40]. They also observed that GF mice spent longer with an object or an empty chamber than with another mouse, which is considered abnormal behavior for a sociable animal. Research has also demonstrated that colonization of the GF mice partially normalizes these behavioral impairments[40].

Oxytocin is well known to influence social behavior[41] and evidence indicates that its levels are closely regulated by the gut microbiota[42]. In fact, last year Desbonnet *et al*[21] showed that depletion of the gut microbiota as of early adolescence reduces oxytocin expression in the adult brain. Furthermore another recent study demonstrated that a single probiotic bacteria (a strain of the species *Lactobacillus reuteri*) can modulate oxytocin levels and reverse autism-related behavior, raising the possibility of influencing social behavior by targeting the gut microbiota[42].

Autism spectrum disorder (ASD) is often associated with gastrointestinal co-morbidities and recent studies have shown changes in the gut microbiota of autistic children, including shifts in levels of Bacteroidetes and Firmicutes phyla with the abundance of Clostridium, establishing a strong link between gut microbiota and ASD[43,44]. Research also reports an increase in microbiota diversity associated with autism in children with the abundance of Bacteroidetes found to be linked with severe autistic cases[44]. Other gut commensals found to be altered in autism belong to *Bifidobacterium, Lactobacillus, Prevotella* and

Ruminococcus genera[44] although these associations do not necessarily imply causality. In addition, a significant increase in SCFAs in fecal samples from autistic children has been recorded, providing a further indication for a role of an altered microbiota composition or function in this neurodevelopmental disorder[45]. However, the role of SCFAs in ASD is not fully understood. For instance, administration of butyrate has been shown to improve repetitive symptoms in a murine model of ASD[46] whereas intra cerebroventricular infusions of propionic acid induces autistic-like behaviors in rats[47], thus suggesting SCFAs play differential roles in mediating ASD behavior. Therefore, further research is warranted to delve further into the role of SCFAs in autism.

In humans, prenatal exposure to the mood stabilizer valproate is a major risk-factor for autism[48] and de Theije *et al*[49] have shown that the autism-like behavioral changes that occur in mouse models of valproate exposure are coincident with changes in microbiota composition.

In addition, maternal obesity is associated with neurodevelopmental disorders in offspring, including autism spectrum disorder[50] and, in line with this observation, mice with induced obesity by a maternal high-fat diet (MHFD) show social behavioral deficits due to alterations in the gut microbiota[51]. Buffington *et al*[51] recently observed that the diversity of the microbiota in MHFD offspring was reduced, showing a remarkable reduction in *Lactobacillus* spp. compared with the abundance found in the offspring of animals from mothers on a regular diet. Furthermore, it was demonstrated that treatment with a *Lactobacillus reuteri* strain not only augmented levels of oxytocin, improving social behavior, but also ameliorated synaptic dysfunction in MHFD offspring[51].

Schizophrenia is another complex heterogeneous behavioral disorder characterized by abnormal social behavior, often associated with additional mental health problems such as anxiety disorders and major depression[52]. To date the genomic analysis of schizophrenia has been limited, with the replicated genetic findings representing just a fraction of schizophrenia heritability[53]. Furthermore, there is evidence for the important role that different environmental factors play in its development. In fact, several epigenetic mechanisms, in particular methylation of genes involved in neurotransmission, histone modifications and the action of ncRNAs, may also predispose individuals to schizophrenia. There are indications, such as the fact that dopamine, the key neurotransmitter associated with schizophrenia pathophysiology and treatment, is produced by microbes, and the increased gastrointestinal inflammation associated with schizophrenia, which strongly suggest that gut microbiota are involved in the risk of development schizophrenia or its manifestations[54]. In addition, several studies have found an association between the intake of antibiotics and an increased incidence of psychiatric disorders such as schizophrenia, perhaps due to alterations in the microbiota[55]. However, to date there are not any published studies investigating the role of the gut microbiome in schizophrenia.

Attention deficit hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterized by inappropriate levels of hyperactivity, difficulty in controlling behavior and/or attention problems. Although ADHD is currently one of the most frequently studied disorders in children and adolescents, the exact mechanisms that predispose individuals are still unknown, though both genetic and environmental factors seem to be involved[56]. Various factors associated with the risk of developing ADHD and/or linked to different ADHD manifestations have also been linked to shifts in gut microbiota composition, suggesting a link between the microbiota and the disorder. In addition, evidence from preliminary human studies suggests that dietary components modulating gut microbiota may also influence ADHD development or symptoms. Therefore, recently, after reviewing the literature, we argue[57] that genomic studies in ADHD should include studies of the gut microbiota.

Microbiota, stress response and depression

Most organisms are equipped with biological machinery able to muster a defensive response to stressful stimuli. In response to stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated and corticosterone releasing factor (CRF) is released from paraventricular neurons of the hypothalamus. CRF stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary, which in turn induces the synthesis and release of glucocorticoids from the adrenal cortex: cortisol in humans and corticosterone in animals. Studies in GF mice have revealed that the microbiota influences the development of the HPA axis and thus the stress response. Animals raised in a sterile environment from birth exhibit inflated HPA axis activity with elevated ACTH and corticosterone levels in response to a stressor[22]. Interestingly, HPA axis activity is normalized after colonization with commensal bacteria from control mice[25].

Although studies investigating the effects of prebiotic or probiotic supplements on stress behavior in humans are limited, they indicate the role of gastrointestinal microbiota in stress and emotional responses. Likewise, a probiotic combination (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175)[58] and a prebiotic (galactooligosaccharide)[59] have proven effective in increasing the subject's resilience to stress and improved emotional responses in healthy subjects.

Depression is a stress-related mood disorder associated with a disrupted HPA axis, and evidence suggests that the gut microbiota play a key role in modulating depression[60]. In fact, an increase in alpha diversity of the gut microbiota has been reported in individuals with depression compared to a healthy

control group. Furthermore, patients with depression show significantly lower numbers of *Bifidobacterium* and *Lactobacillus* compared to control subjects[61]. In addition, a more recent study shows that patients with major depression have altered microbiota compared to normal subjects, with a significant increase in the genus *Eggerthella*, *Holdemania*, *Gelria, Turicibacter, Paraprevotella* and *Anaerofilm,* whereas reductions in *Prevotella* and *Dialister* were observed[62]. Another recent study also reported a negative correlation between *Faecalibacterium* spp. and the severity of depressive symptoms[62]. Moreover, researchers have demonstrated that when the microbiota from patients with major depression is transferred to microbiota-depleted animals, the behavioral and physiological features characteristic of depression are also transferred, supporting a link between dysbiotic microbiota and depression[63]. A recent study published in Science reported a correlation between a more diverse gut microbiota composition and depression in humans after investigating the gut microbiomes of 1135 participants from a Dutch population cohort using deep sequencing[64].

Different diets have been suggested to have either positive or negative effects on depression. For instance, a Western diet seems to be associated with an increased risk of depression, whereas the Mediterranean diet seems to reduce the onset of depression. Furthermore, studies in human and animal models have shown an association between depletion of omega-3 polyunsaturated fatty acids and major depression, suggesting the role of diet in depression onset[65].

Different probiotic treatments have displayed efficacy in the reduction of depressive-like behaviors in animal models. For instance, administration of a probiotic cocktail comprising of *Lactobacillus rhamnosus* and *Lactobacillus helveticus* strains have been shown to ameliorate depressive-like behavior and normalize corticosterone levels in a maternal-separation animal model. Moreover, administration of *Lactobacillus rhamnosus* reduced depression and anxiety-related behavior. Also there is evidence for the association between different strains of the genus *Bifidobacterium* and potential antidepressant-like behavior in animals. Treatment with a strain of *Bifidobacterium infantis* attenuated depression, showing increased mobile episodes during the forced swim test in maternally separated rats. A similar effect was also observed with strains of *Bifidobacterium longum* and *Bifidobacterium breve* on depression and anxiety-related behavior in rodents[60].

Gut microbiota and neurodegenerative conditions

Over a century ago, the Nobel Prize Elie Metchnikoff already suggested that the microbial communities within the gastrointestinal tract had an influence on human health. The Russian scientist Metchnikoff observed that people lived longer in parts of Bulgaria and Eastern Europe because of the high consumption of fermented dairy products containing lactic acid bacteria, and suggested that complementing the diet with lactic acid bacteria would have health benefits, including longevity[66]. The finding that germ-free mice live longer than their conventionalized controls was also reported many years ago[67,68], supporting a link between microbiota and senescence. Nowadays, we know that the gut microbiota undergoes a dynamic change during aging[69]. It is of interest that bifidobacteria numbers decrease with age, while those of clostridia increase[70]. Age-related gut microbiota composition changes have also been correlated with health outcomes in the elderly, such as frailty[71], with microbial diversity being an important feature linked to health maintenance as we age[18]. However, major shifts in diet of the elderly could partly be responsible for the dramatic changes in microbiota composition and their association with health-relate outcomes[72]. This also suggests there is a chance of redressing the balance by dietary-based interventions in the elderly.

Aging can weaken gastrointestinal barrier function and promote a proinflammatory phenotype involving the microbiota[73]. Another consequence of ageing is the progressive leakiness of the blood-brain-barrier (BBB), whose integrity also seems to be dependent on gut microbiota composition[74]. SCFAs produced by gut microbiota components are considered key metabolites mediating such effects. Stress is one of the lifestyle factors that can negatively impact BBB permeability[75] and accelerate the "inflamm-aging" processes linked to age-related diseases[76]. Therefore, understanding how the gut microbiota or their components influence such processes is now worthy of attention.

Parkinson's disease (PD) is a neurodegenerative disorder that represents a growing health concern in the elderly. It is characterized by neuroinflammation and loss of midbrain dopaminergic neurons, as well as by a characteristic pattern of abnormal movements with a number of non-motor symptoms[77]. It has also been observed that alterations in bowel function, mainly constipation, often precede the onset of prototypical motor symptoms associated with PD. While genetics plays an important role in the risk of developing the disease, environmental factors and gene-environment interactions also contribute to the risk for developing the disorder[78]. In fact, evidence suggests that gut microbiota is an important environmental factor related to the risk of PD[78-81].

Interestingly, a recent study sequenced the gut microbiota in patients with PD and controls[81]. The authors of this study compared 72 patients and 72 matched controls, confirming a major reduction in the levels of Prevotellaceae in PD patients. They also observed and described a positive correlation between the levels of Enterobacteriaceae and the severity of postural instability and gait difficulty[81], suggesting the role of the gut microbiota in the PD phenotype. Another study showed that at the taxonomic level of genus,

putative "anti-inflammatory" butyrate-producing bacteria from the genera *Blautia, Coprococcus*, and *Roseburia* were significantly more abundant in feces of controls than PD patients. On the other side, in this study it was also reported that bacteria from the genus *Faecalibacterium* were significantly found more abundant in the mucosa of controls than PD patients, whereas putative "pro-inflammatory". Proteobacteria of the genus *Ralstonia* were significantly more abundant in mucosa of PD patients than controls[81].

Intriguingly, another recent study confirmed the recently reported association between PD and the reduced abundance of butyrate-producing bacteria, and also demonstrated a reduction in the relative SCFAs concentration in PD compared with the abundance observed in controls, suggesting a role for SCFAs in PD[78]. However, prospective longitudinal studies in subjects at risk of PD are still required to further elucidate the causal role of the gut microbiota and microbial products in the development of PD and its manifestations.

Very recently it has been reported that gut microbiota is involved in motor deficits and neuroinflammation in a model of PD, suggesting that the changes in the gut microbiota represent a risk factor for PD[78]. This study revealed that under GF conditions, or antibiotic-related bacterial depletion, transgenic animals overexpressing human α-synuclein (αSyn) (an abundant protein in the human brain involved in neurotransmitter release) displayed reduced microglia activation, αSyn aggregates and motor deficits (neuropathological features of PD) compared to animals with a complex microbiota. Conversely, they showed that treatment with SCFAs restored all major features of PD in GF mice. Furthermore, this study demonstrated that transplanting gut microbiota from PD patients into genetically susceptible mice (αSyn-overexpressing mice) enhances physical impairments when compared to microbiota transplant from healthy human donors[78].

Alzheimer's disease (AD) and vascular dementias are the most common causes of cognitive decline in ageing populations in Western countries[82]. The deficit in synaptic plasticity is one of the many changes occurring with age. Specifically, the typical model of plasticity shows a reduction in the hippocampus long-term potentiation (LTP) of the middle-aged, but most dramatically in aging rats[83]. A recent study has investigated whether the age-related deficit in LTP might be attenuated by changing the composition of intestinal microbiota with VSL#3, a probiotic mixture comprising 8 Gram-positive bacterial strains. The study showed that the age-related deficit in LTP was attenuated in VSL#3-treated aged rats and this was accompanied by a modest decrease in markers of microglial activation and an increase in expression of BDNF and synapsin[84]. However, although these findings support the fact that intestinal microbiota can be manipulated in order to positively impact neuronal function modulating microglial activation, at the moment we still need to be cautious and to investigate further the different probiotics that could be used to modify the microglial maturation and function.

Surprisingly, so far there is a lack of detailed analyses of the microbiota of patients with AD[85]. However, in metabolic syndrome, type 2 diabetes and obesity, which are risk factors for AD[86], there is an alteration in the gut microbiota[87,88]. More recently, a research study using a mouse model of AD has implicated the microbiota in the accumulation of amyloid plaques[89], and there is also evidence suggesting that gut microbiota might be directly linked to dementia pathogenesis by triggering metabolic diseases and low-grade inflammation[90]. Different mechanisms may explain the link between gut microbiota alterations in obesity and T2D and the development of AD. For example, different studies have indicated that an altered gut microbiota linked to obesity increases intestinal permeability and contributes to systemic inflammation leading to insulin resistance and T2DM[91]. In turn, insulin resistance and T2DM is a risk factor for development of AD. Furthermore, the vascular effects of obesity and T2D, related to changes on the gut microbiota, also appear to play an important role in the development of AD[92]. A leading hypothesis on the pathophysiology of AD is the mis-metabolism of amyloid precursor protein. The Aβ peptide is derived from amyloid precursor protein by sequential cleavages of different proteases. The activity of these proteases involved in the generation of Aβ peptide is highly regulated by the inflammation, being the latter modulate as already mentioned by the gut microbiota. In fact, BACE1 enzyme is essential for the generation of β-amyloid and Interleukin 1β, considered as a risk factor for AD development, has been observed to aggravate plaque formation by induction of BACE1 expression[93].

However, although it has been suggested that alterations on gut microbiota observed in diabetes and obesity may be linked to the risk of developing AD, there is a need of further work to elucidate the specific gut's microbes and the mechanisms involved in the link between obesity, T2D and AD.

Dysregulation of normal microglial functions such as synaptic pruning and regulation is increasingly found to be implicated in diseases associated with cognitive deficits[12,94]. Microglia cells are also essential for clearance of debris, plaques and aggregates, thereby playing a fundamental role in neurodegenerative amyloid disorders, including Alzheimer's, Huntington's and Parkinson's diseases, each associated with a distinct amyloid protein. Therefore, targeting dysregulated microglial functions represents a therapeutic opportunity for treating these disorders[95,96]. Although the mechanisms that ultimately lead to neurodegeneration are different in each neurodegenerative disease, chronic inflammation that may be modulated by the gut

microbiota is typically a prominent feature in the progressive nature of neurodegeneration[12].

CONCLUSION

It is becoming evident that brain development and function are dependent on the diversity and structure of the gut microbiota and may, therefore, influence mental health. This hypothesis is based mainly on animal trials and a few observational human studies associating gut microbiota alterations with mental disorders, including depression, autism and PD. This notion has been further supported by recent transplantation experiments where the gut microbiota has been shown to transfer a behavioral phenotype or the disease features to the recipient animal, providing stronger evidence for a causal relationship. However, longitudinal studies in humans are still needed to investigate whether the gut microbiota changes in subjects with different neuropsychiatric disorder can contribute to disease onset and the role of other interacting factors such as the diet. So far various communication routes between the microbiota and brain have been identified, including immune, endocrine and neural pathways. Thus, interventions with pro and pre-biotics in animal models have shown that the microbiota could play a role in mental health by regulating inflammatory and endocrine secretions, synthetizing neuroactive compounds and interacting with the vagal nerve. Greater understanding of the precise underlying mechanisms is required to develop a clear rationale for conducting microbiota-based interventions in humans. In particular, inflammation seems to be a critical pathophysiological feature of mental disorders and, therefore, a potential target for microbiota-based interventions. Nonetheless, further knowledge is needed on how microbiota signals generated in the gut can impact on microglial activation and neuroinflammation.

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P- Reviewer: Cibor D, Dutta AK, Li HD **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Huang Y

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5499-5507

DOI: 10.3748/wjg.v23.i30.5499

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

ORIGINAL ARTICLE

Basic Study

Everolimus halts hepatic cystogenesis in a rodent model of polycystic-liver-disease

Frederik Temmerman, Feng Chen, Louis Libbrecht, Ingrid Vander Elst, Petra Windmolders, Yuanbo Feng, Yicheng Ni, Humbert De Smedt, Frederik Nevens, Jos van Pelt

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Institutional animal care and use committee statement: All animal experiments were approved by the Ethical Committee for animal welfare (KU Leuven, P164/2010).

Conflict-of-interest statement: None of the authors report a potential conflict of interest regarding this work.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: February 27, 2017
Peer-review started: March 2, 2017
First decision: April 5, 2017
Revised: May 16, 2017
Accepted: June 1, 2017
Article in press: June 1, 2017
Published online: August 14, 2017

Abstract

AIM

To develop a MRI-based method for accurate determination of liver volume (LV) and to explore the effect of long-term everolimus (EVR) treatment on LV in PCK

rats with hepatomegaly.

METHODS

Thirty-one female PCK rats (model for polycystic-liver-disease: PCLD) were randomized into 3 groups and treatment was started at 16 wk, at the moment of extensive hepatomegaly (comparable to what is done in the human disease). Animals received: controls (n = 14), lanreotide (LAN: 3 mg/kg per 2 wk) (n = 10) or everolimus (EVR: 1 mg/kg per day) (n = 7). LV was measured at week 16, 24, 28. At week 28, all rats were sacrificed and liver tissue was harvested. Fibrosis was evaluated using quantitative image analysis. In addition, gene (quantitative RT-PCR) and protein expression (by Western blot) of the PI3K/AkT/mTOR signaling pathway was investigated.

RESULTS

LV determination by MRI correlated excellent with the *ex vivo* measurements (r = 0.99, P < 0.001). The relative changes in LV at the end of treatment were: (controls) +31.8%; (LAN) +5.1% and (EVR) +8.8%, indicating a significantly halt of LV progression compared with controls (respectively, P = 0.01 and P = 0.04). Furthermore, EVR significantly reduced the amount of liver fibrosis (P = 0.004) thus might also prevent the development of portal hypertension. There was no difference in phosphorylation of Akt (Threonine 308) between LAN-treated PCK rats control PCK rats, whereas *S6* was significantly more phosphorylated in the LAN group. Phosphorylation of Akt was not different between controls and EVR treated rats, however, for *S6* there was significantly less phosphorylation in the EVR treated rats. Thus, both drugs interact with the PI3K/AkT/mTOR signaling cascade but acting at different molecular levels.

CONCLUSION

Everolimus halts cyst growth comparable to lanreotide and reduces the development of fibrosis. mTOR-inhibition should be further explored in PCLD patients especially those that need immunosuppression.

Key words: Fibrocystic liver disease; mTOR inhibitor; Somatostatin analogue; Liver volume measurement; Magnetic resonance imaging

Core tip: The continuous increase of liver cysts volume in polycystic-liver-disease (PCLD) leads to extensive hepatomegaly and portal hypertension, an indication for liver transplantation. The effect of mTOR-inhibition on liver volume (LV) in PCLD is unclear. We developed an accurate, non-invasive, MRI-based method to determine LV in a PCLD rat model. When treatment is started at the moment of extensive hepatomegaly (as in humans), the mTOR inhibitor everolimus halt disease progression and also of the development of fibrosis in this model. We speculate that everolimus, given after kidney transplantation in patients with PCLD, can prevent the development of symptomatic hepatomegaly.

Temmerman F, Chen F, Libbrecht L, Vander Elst I, Windmolders P, Feng Y, Ni Y, De Smedt H, Nevens F, van Pelt J. Everolimus halts hepatic cystogenesis in a rodent model of polycystic-liver-disease. *World J Gastroenterol* 2017; 23(30): 5499-5507 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5499.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5499

INTRODUCTION

Polycystic liver disease (PCLD) is a fibrocystic liver diseases, a group of genetic disorders in which cysts occur either only in the liver, like in autosomal dominant PCLD, or in liver and the kidneys as in autosomal dominant polycystic kidney disease (ADPKD)[1,2]. In these patients due to the continuous increase in volume and number of cysts, the liver enlarges and may become disabling and in advanced stages, the patients develop portal hypertension[3].

The rat *Pck* gene is orthologous to the human *PKHD1* gene and responsible for Autosomal Recessive Polycystic Kidney Disease. The animals have a splicing mutation in the Pkhd1 gene encoding fibrocystin/polyductin (FPC). *FPC* and polycystin-1 and -2, -proteins mutated in human ADPKD-, are co-localized to the primary cilium of the cholangiocytes. The PCK rat is used worldwide as model to study PCLD[4-7].

Two key signaling pathways have been implicated in the increased proliferation of PCK cholangiocytes leading to cyst formation. First, the defective ciliary structure in the cholangiocytes and integrated sensory/transducing functions result in a decreased intracellular Ca^{2+} and increased cytosolic cyclic adenosine monophosphate $[cAMP]_{cyt}$, causing cholangiocyte proliferation, abnormal cell-matrix interactions, and altered fluid secretion. Basal levels of cAMP are maintained by the orchestration of multiple factors in which somatostatin receptors (*SSTRs*) play an important role. Octreotide, a somatostatin analogue, with high binding affinity for *SSTR2* and *5*, decreases $[cAMP]_{cyt}$ and reduces liver-cyst volume in the PCK rat. Both lanreotide (LAN) and octreotide reduce liver volume (LV) in patients with PCLD[8-13].

The other important pathway involves the mammalian target of Rapamycin (*mTOR*). *mTOR* is a serine/threonine kinase present in two distinct complexes. The first is *mTOR* complex 1 (mTORC1), composed of *mTOR, Rptor* (*Raptor*: Regulatory Associated Protein of mTOR, Complex 1), *GβL*, and *DEPTOR*. It is a master growth-regulator that senses and integrates diverse nutritional and environmental factors. The second complex, *mTOR* complex 2 (mTORC2), is composed of *mTOR, Rictor, GβL, Sin1,*

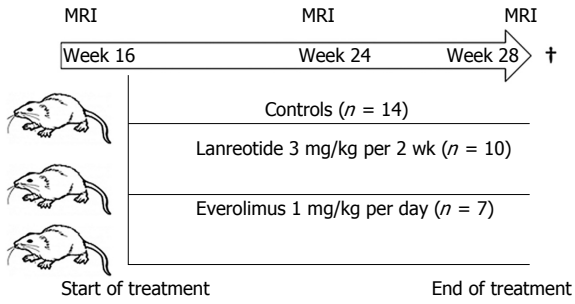


Figure 1 Study design. Female PCK rats were obtained at age week 10. At week 16, a first MRI was performed to calculate the liver volume. Animals were randomly assigned to one of the groups and treatment was started. At week 24 and week 28 a new MRI was performed, after the last measurement the animals were sacrificed and tissue collected for protein and gene assay.

PRR5/Protor-1, and *DEPTOR*. mTORC2 promotes cellular survival by activating *Akt*, regulates cytoskeletal dynamics and controls ion transport and growth[14,15]. Increased activation of the Phosphatidylinositol-4,5-bisphosphate 3-kinase/AkT/ mTOR (*PI3K/Akt/mTOR*) pathway has also been shown to be involved in the cystic proliferation of cholangiocytes of the PCK rat. Sirolimus, an mTOR inhibitor, delayed cyst growth in the Han:SPRD rat model and in a mouse model of ADPKD[16,17]. In human ADPKD patients, sirolimus reduced LV in patients who underwent kidney transplantation in one study[18]. However, the evidence of a beneficial effect of mTOR inhibitors to reduce LV in the PCK rat and in prospective studies in humans is not robust[19,20].

From preclinical studies performed in the PCK rat and from human PCLD data, is it clear that the hepatic cystic disease progression shows large inter-individual variability. To overcome this we explored the value of non-invasive repeated measurements of LV by MRI in the PCK rat.

The aims of the present study were therefor: (1) to develop an MRI-based method to evaluate accurately changes in LV in a rat model of PCLD; and (2) to investigate the long-term efficacy of everolimus (EVR) on liver disease when administered starting at the moment of already marked hepatomegaly, a situation mimicking the clinical situation.

MATERIALS AND METHODS

Experimental protocol

Thirty-one 10-wk-old female PCK rats (Charles River, France) were used for this study. This rat model is derived from a Crj:CD (Sprague Dawley) rat strain, originating in Japan[4-7]. The animals were housed in an environment with normal humidity and a 12/12 daylight cycle receiving a normal diet. They were randomly assigned to three groups: (1) controls; (2) LAN as positive control; or (3) EVR. At week 16, when the animals had developed extensive hepatomegaly, baseline MRI was performed. The next day, therapy was started and continued for a period of 3 mo. No animals died or were excluded for other reasons over the entire study period. At indicated times (week 16, 24 and 28), serial MRI were performed (Figure 1). All rats were sacrificed the day after their last MRI at week 28 using Nembutal anesthesia. After vertical laparotomy, the hepatic hilum and hepatic veins were clipped simultaneously to avoid change in LV by loss of blood. Livers were removed, weighted and the *ex vivo* LV was determined using a graduated glass cylinder filled with saline 0.9% at 37 ℃ (accuracy of 1 mL). Tissue samples were stored for molecular analysis in Trizol (Invitrogen/Life Technologies, United States), snap-frozen for protein analysis and fixed with formalin for histology.

All animal experiments were approved by the Ethical Committee for animal welfare (KU Leuven, P164/2010).

Study drugs

Lanreotide 3 mg/kg was administered every 2 wk intramuscular (somatuline, gift from Ipsen Pharma; Merelbeke, Belgium). Everolimus oral solution 1 mg/kg per day (Certican®; gift from Novartis Pharma; Basel, Switzerland) was administered *via* the drinking water without further additives. Dosages were chosen based on previous published data[10,12,20]. We used black drinking bottles to ensure light protection in the EVR group. Drug solutions were freshly prepared every morning and adjusted to body weight and fluid intake once weekly.

Liver volumetry

The PCK rats were anaesthetized with Isoflurane 2% and placed in a human wrist coil. MRI scanning (1.5T MRI scanner; slice thickness 0.3 cm; inter-slice gap: 0.03 cm) for liver volumetry was performed by 3 radiologists, blinded for the study groups; on the day before the start of treatment (week 16), at week 24 and at week 28 (*i.e.*, 8 and 12 wk after the start of therapies). T1 and T2 weighted MR images were acquired. Liver area was measured and summed for all slices of T2 weighted images by using a built-in freehand region of interest (Figure 2A). Then, the LV was calculated using the following formula: LV = Σ liver area on each slice × (slice thickness + gap).

Fibrosis

Liver samples were fixed in buffered formalin (4%), washed in PBS and embedded in paraffin. Picrosirius red collagen staining was performed to measure fibrosis. All liver biopsies were analyzed by an expert liver pathologist, blinded for the groups. Fibrosis was assessed using Olympus Stream image analysis software 1.9 Software following image acquisition using a light microscope and color digital camera (Olympus CMOS camera SC30 Münster, Germany). Fibrosis was scored by 2 independent researchers and

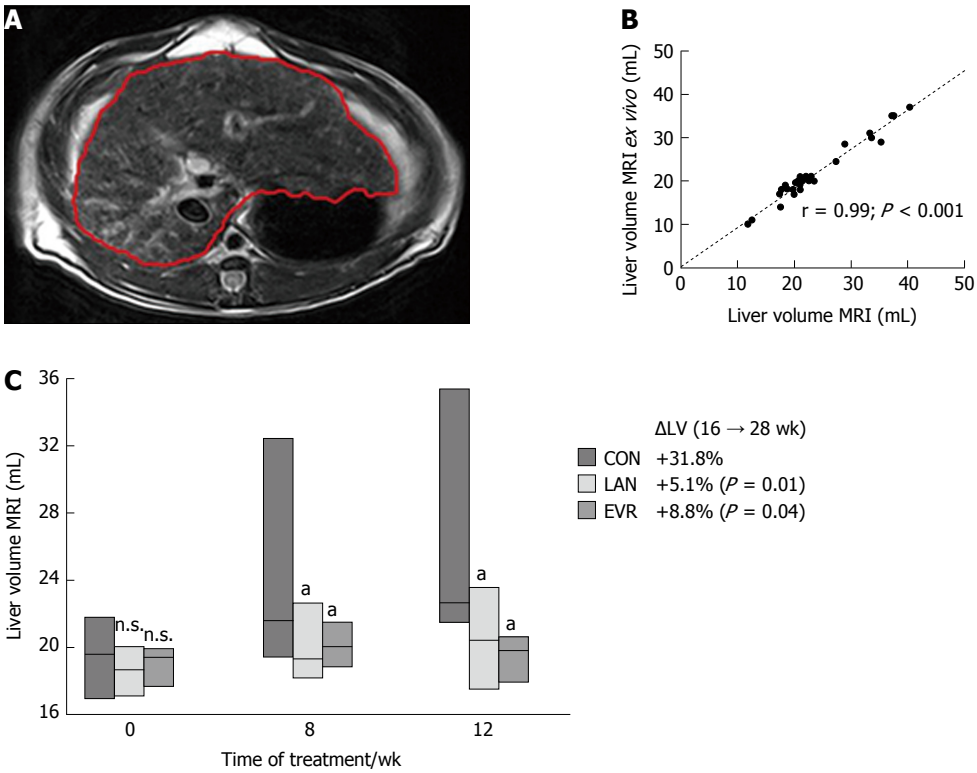


Figure 2 Liver volume measurements. A: Demonstration of liver volume measured by MRI on each liver-containing slice of T2 weighted images. The liver margin is contoured manually with the red line; B: Correlation between liver volume calculated from MRI scan and liver volume measured *ex vivo*. Pearson correlation (r) is given together with significance; C: Liver volume (mean ± SE) determined by MRI for the different groups: CON (controls, n = 14), LAN (n = 10) and EVR (n = 7), measured at start of treatment, and after 8 and 12 wk. [a]$P < 0.05$ *vs* animals in control group at corresponding time, NS: Not significant, *vs* control. The percentual change in LV (ΔLV) over the 12 wk experimental period is shown together with the P value *vs* control group. LAN: Lanreotide; EVR: Everolimus.

expressed as percentage of total liver parenchyma from 4 random selected samples per animal.

Western blotting

Liver samples stored at -80 ℃ were homogenized using Tissue Lyzer LT (Qiagen) in RIPA buffer at 4 ℃ (50 mmol/L Tris pH 8.0, 150 mmol/L NaCl, 0.01% sodium dodecyl sulfate, 1% NP-40 (nonionic polyoxyethylene surfactant), 0.5% sodium desoxycholaat, 1 mmol/L 1-phenylmethylsulfonyl fluoride) containing protease inhibitor mix (Complete Protease Inhibitor Cocktail, Roche Applied Science, Penzberg, Germany). Protein concentrations were assessed with the BCA-kit (Abcam, Cambridge United Kingdom) and the protein in the lysates were adjusted to the same concentration (40 μg/20 μL). SDS sample buffer (62.5 mmol/L Tris, 10% glycerol, 2% sodium dodecyl sulfate, 0.05% Bromophenol blue and β-mercaptoethanol) was added, samples were boiled for 5 min and separated on miniprotean TGX anykD gel from Biorad (Biorad, Hercules, CA, United States). After electrotransfer to nitrocellulose membrane and blocking in Phosphate-buffered saline (PBS) containing 0.1% Tween and 5% non-fat dried milk, the membranes were overnight incubated with the primary antibody in PBS supplemented with 0.1% Tween and 5% non-fat dry milk powder. Antibodies used were: anti-P-S6Rp (Ser235/236, #4858), anti-Tot S6Rp (#2217), anti-P-Akt (Thr308,#) anti-Tot Akt (#2967), all purchased from Cell Signaling Technology (Beverly, Massachusetts, United States), and β-actin (Sigma-Aldrich, St. Louis, MO, United States) as loading control. Thereafter, the corresponding secondary horseradish peroxidase-coupled antibodies were applied to the membranes for one hour (Dako, Heverlee, Belgium). After addition of enhanced chemiluminescence reagent (Pierce ECL Western Blotting Substrate, #32106, Thermo Scientific/Life Technologies, Carlsbad, CA, United States), digital detection was performed using ChemiDoc™ imaging system with Image Lab™ image acquisition software (Biorad). Expression of P-S6Rp, total S6Rp, P-Akt, and total Akt was normalized to β-actin levels. For comparison of different blots, a pool of liver homogenates of 28-wk-old Sprague Dawley rat livers was placed on each gel as internal control.

Quantitative Real-Time polymerase chain reaction

Gene expression was assessed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). RNA was isolated from tissue stored in Trizol with the RNeasy Kit (Qiagen, Chatsworth, CA, United States) according to the manufacturer's instructions. One microgram of cellular RNA was transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen/Life Technologies, United States). The PCR reaction was carried out in a mixture

Table 1 Real-time polymerase chain reaction primer probes used in this study

Gene	Full name	Assay ID applied biosystems
SSTR2	somatostatin receptor 2	Rn01464950_g1
SSTR5	somatostatin receptor 5	Rn02535169_s1
mTOR	mechanistic target of rapamycin (serine/threonine kinase)	Rn00693900_m1
Rptor	regulatory associated protein of mTOR, complex 1	Rn01464431_m1
B2M	beta-2 microglobulin	Rn00560865_m1

that contained appropriate sense- and anti-sense primers and a probe in Taqman Univeral PCR Master Mixture (Applied Biosystems, Foster City, United States) (Table 1). β-2 microglobulin (*B2M*) was used as house-keeping gene. Each sample was assayed in duplicate on an A7500 Fast Real-Time PCR System (Applied Biosystems). The mean ΔCt value (with SE) *vs* the reference (*B2M*) was calculated.

Statistical analysis

Data were analyzed using MedCalc version 14.12.0 (Medcalc, Ostend, Belgium: http://www.medcalc.org). Descriptive statistics including mean and SE for continuous variables were computed or median with IQR (interquartile range) (25%-75%) as appropriate. Differences in continuous variables between treated and non-treated rats were investigated using, One Way ANOVA, *t*-test for independent samples or the Mann Whitney *U* test, as appropriate. Repeated measurements ANOVA and serial measurements were used to compare paired observations between groups, as appropriate. To assess correlations, non-parametric testing Pearson correlation coefficient was determined. Bland-Altman plots assessed agreement in accuracy of the techniques. To compare percentages between groups, χ^2 was used. All *P* values resulted from two-sided statistical tests, and $P < 0.05$ is considered significant.

RESULTS

Liver volume

To validate MRI as a tool, we determined LV by two methods. (1) *In vivo* LV was determined by MRI (Figure 2A); and (2) To measure the LV *ex vivo,* we removed the liver at the end of the experiment (week 28, corresponding with 3 mo of treatment). Pearson correlation between *in vivo* LV (MRI) and *ex vivo* LV was excellent ($r = 0.99$, $P < 0.001$) (Figure 2B) and that allows us to use MRI as a reliable method to explore LV. At baseline rat body weight (gram) in the 3 groups: controls, LAN and EVR were not different, respectively: 316 (SE: 3.2); 318 (SE: 5.6) and 299 (SE: 2.7). Also at baseline, the median LV's (range) for controls, LAN and EVR, were not different, respectively: 19.6 mL (17.0; 21.7); 18.7 mL (17.0; 20.0); 19.4 mL (17.8; 20.6) ($P = 0.754$). The mean of the relative increase in LV (95%CI) in the 3 groups after 12 wk of treatment (from week 16 to week 28) was respectively: +31.8% (19.0;44.0); +5.1% (-12.0; 23.0) and +8.8% (-1.7; 19.0). Both treatment groups (LAN, EVR) significantly halted LV progression compared with controls ($P = 0.01$ and $P = 0.04$). The absolute LVs at different times are given in Figure 2C. There was no significant difference in effect on LV between LAN and EVR after 8 wk or 12 wk of treatment.

Fibrosis

Fibrosis, scored independently by 2 researchers, showed excellent agreement with a difference of 0.5% (95%CI: -0.2-1.3). In the 14 control PCK rats [mean percentage fibrosis: 14.7% (SE: 1.8)], we found a strong Pearson correlation (r) between LV (MRI) and the relative amount of fibrosis ($r = 0.93$; $P < 0.0001$) (Figure 3A). EVR significantly suppressed the development of fibrosis in PCK rats ($P = 0.004$) whereas LAN showed a trend to reduction ($P = 0.095$) (Figure 3B). Representative histological images of picrosirius red staining are shown in Figure 3C.

Protein expression

Expression of components of the PI3K/Akt/mTOR pathway was investigated. In the control animals, we observed a very strong correlation between the p-Akt/Akt phosphorylation ratio and the LV ($r = 0.7$, $P < 0.003$) while the *p-S6/S6* ratio correlated only moderately ($r = 0.47$, $P = 0.088$) (Figure 4A).

There was no difference in phosphorylation of *Akt* (Threonine 308) between LAN-treated PCK rats control PCK rats, whereas *S6* was significantly more phosphorylated in the LAN group. The *p-S6/S6* ratios for LAN and controls were respectively: 2.01 (0.98; 2.53) and 0.33 (0.16; 0.76) ($P < 0.001$). The level of phosphorylation of Akt at Threonine 308 was not different between controls and EVR treated rats. However, *S6* was significantly less phosphorylated in the EVR treated rats (Figure 4C and D).

These observations indicate that both drugs interact with the PI3K/AkT/mTOR signaling cascade but acting at different molecular levels (Figure 4B), both mechanisms leading to halting of the disease in this model of PCLD.

Gene expression

LAN and EVR treated rats showed a significant lower gene expression of *SSTR2* while expression of *SSTR5* was increased (borderline significant) in the LAN treated rats (Figure 5). EVR treatment resulted in a decreased *SSTR5* gene expression compared to LAN treated animals. After 12 wk of treatment, we observed an increased gene expression of *mTOR* but not of *Rptor* in the EVR treated rats *vs* controls. LAN treatment did not affect gene expression of *mTOR*. Demonstrating again that both drugs have different

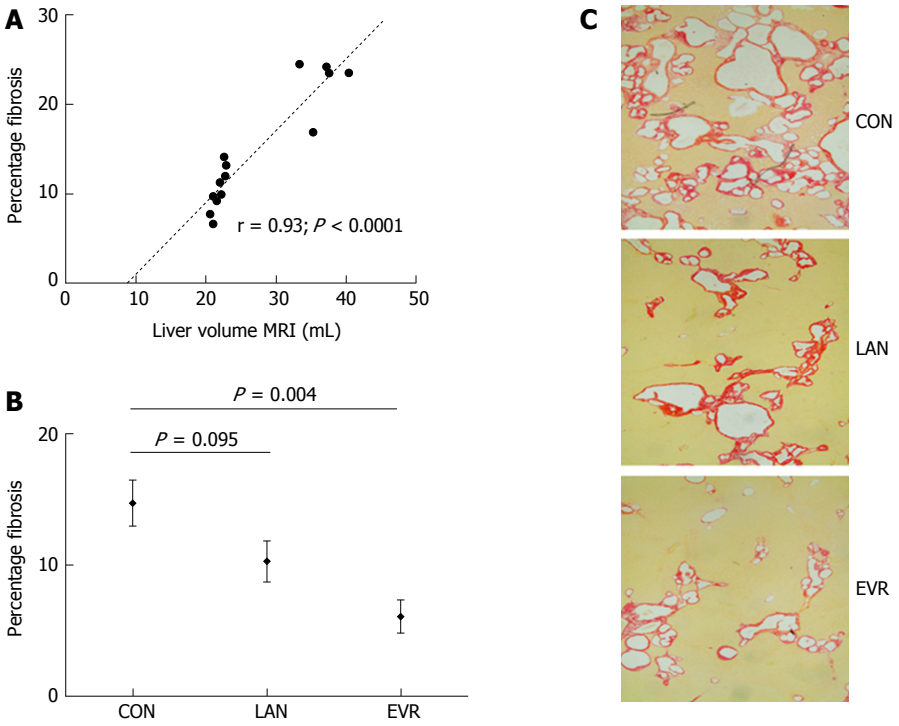


Figure 3 Liver fibrosis in PCK rats and effect of treatment with lanreotide or everolimus. Picrosirius red collagen staining was performed on formalin fixed, paraffin embedded liver tissue, staining was assessed using Olympus Stream image analysis software 1.9 Software. Fibrosis was expressed as percentage of total liver parenchyma from 4 random selected samples per animal. A: Pearson correlation (r) and significance for liver volume with fibrosis in control animals; B: Amount of fibrosis relative to total parenchyma in the livers of PCK rats after 12 wk of treatment with LAN or EVR and in controls. Results are given as mean and SE; C: Representative images of the amount of fibrosis by picrosirius red staining in the 3 groups (original magnification × 40). CON: Control; LAN: Lanreotide; EVR: Everolimus.

mechanisms of action on cyst growth.

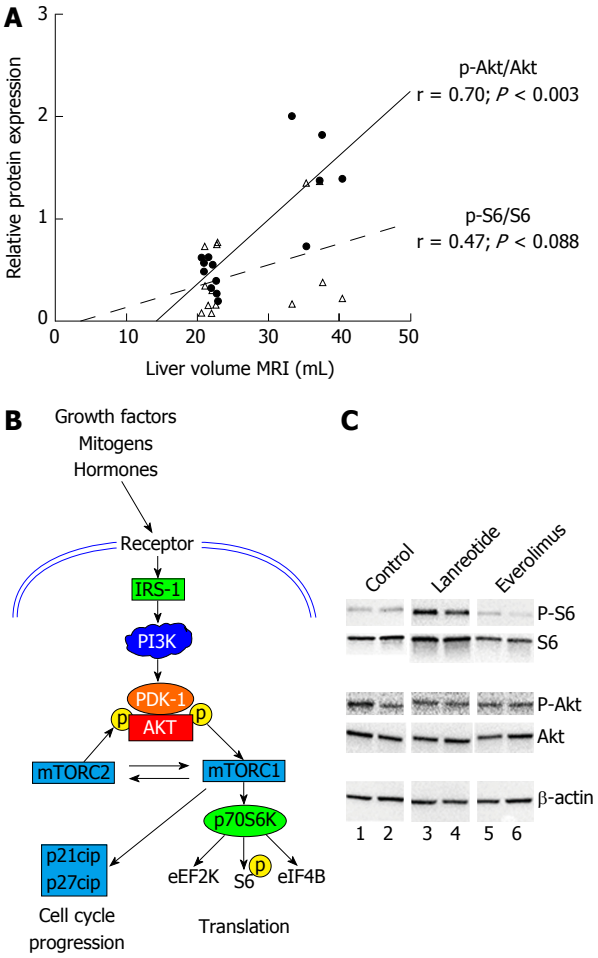
DISCUSSION

Similar to what is observed in human PCLD patients; we found a large inter- and intra-litter variation of the severity of hepatic polycystic disease in the PCK model, which had previously also been found by Mason *et al*[21]. Therefore, there is a great need for a reliable technique that can accurately assess LV in this animal model and further that allows repeated measurements during drug treatment. High Resolution Ultrasonography has recently been used to assess renal cysts in the PCK rats[22]. Also, as an indirect assessment method, T1 relaxation time was proposed as an imaging marker of liver disease for the PCK model[23] but neither of these was used to determined LV. For the current study, we explored MRI T2 weighted images to longitudinally measure LV and to investigate individual responses. We validated this method in the PCK model. We showed that it is accurate and reproducible, with an excellent correlation between LV determined by MRI and *ex vivo* LV determination. The assessment with MRI allowed us to detect changes in the same animal in time accurately. Treatment with LAN was used as positive treatment control and as expected, we observed an increase in LV with placebo and a stabilization with LAN, an observation similar to what is seen in patients with PCLD[10-13].

Important to note is that no animals died or were excluded for other reasons over the entire study period. Using this non-invasive technique, we could drastically reduce the number of animals necessary for this study.

In the present study, we investigated the effect of the mTOR inhibitor EVR on cyst growth since its effect in humans or in the corresponding animal model, is still unclear and controversial due to the limited number of available studies[20,24]. We found that EVR prevented cyst enlargement in the PCK rat model, using a clinical relevant experimental design in which we started the treatment at the moment of severe hepatomegaly, mimicking the clinical situation in humans. The study was restricted to female rats since symptomatic PCLD mainly affects mostly women and because it has been demonstrated that female PCK rats display a more progressive liver enlargement compared to male PCK rats.

Treatment with LAN induced a reduction of *SSTR2* gene expression (in agreement with the expected molecular effect of LAN). In line with previous findings of Masyuk *et al*[8] with octreotide and pasireotide, we also observed upon treatment with LAN a mild reduction in fibrosis compared to untreated animals that might be an indirect effect of lower growth of the liver cysts. With EVR however, we observed a significant reduction of the amount of fibrosis, which may be explained by the well-known direct effect



D

	Controls (n = 14)	Lanreotide (n = 10)	Everolimus (n = 7)	P value (con-lan)	P value (con-evr)
p-Akt/Akt	0.6 (0.4; 1.4)	0.94 (0.68; 1.4)	0.68 (0.5; 0.85)	0.17	0.68
p-S6/S6	0.3 (0.16; 0.76)	2.01 (0.98; 2.5)	0.08 (0.03; 0.1)	< 0.001	0.02

Figure 4 Analysis of protein expression of components of PI3K/AKT/mTOR pathway by Western blot in livers of PCK rats after 12 wk of treatment. A: phosphorylation ratio in untreated animals: correlation between p-Akt/Akt ratio and p-S6/S6 ratio with liver volume. Pearson correlation (*r*) is given together with significance; B: Simplified schematic presentation of PI3K/AKT/mTOR signaling pathway; C: shows two representative samples from each experimental group; D: shows relative expression mean (range) of phosphorylated Akt *vs* total Akt (p-Akt/Akt) and *S6 vs* total *S6* (*p-S6/S6*) with n as number of animals analyzed. Expression of proteins was normalized to β-actin levels.

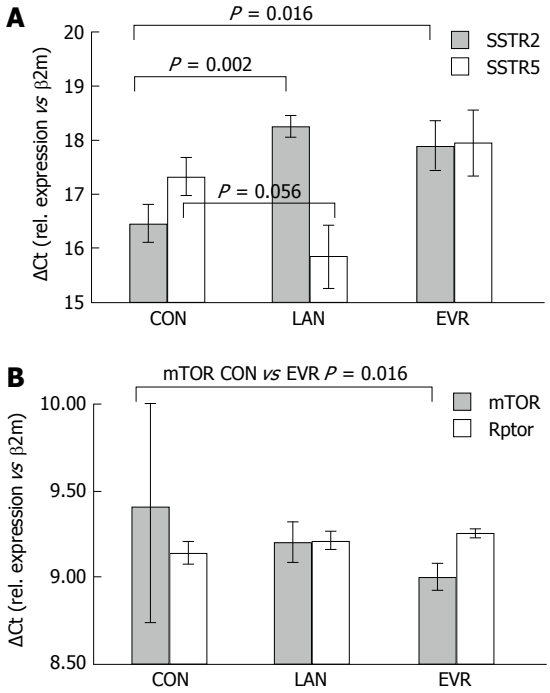


Figure 5 Liver gene expression of target genes for LAN treatment (*SSTR2*, *SSTR5*) and for PI3K/Akt/mTOR pathway (*mTOR*, *Rptor*). A: expression of the Somatostatin receptors *SSTR2* and *SSTR5*; B: expression of *mTOR* and of *Rptor*. The mean for delta Ct value (with SE) is given *vs* that for the reference (housekeeping gene) *β2M*. To be noted, a higher value means a lower expression. CON: Control; LAN: Lanreotide; EVR: Everolimus.

of mTOR inhibition on fibrosis progression[25]. Since PCLD, belonging to the fibrocystic liver diseases, is complicated in a later stage by portal hypertension, the present observations further support an additional potential clinical benefit of this drug on portal hypertension by reducing the amount of fibrosis.

The molecular observations made in this model (the combination of protein and gene expression) indicate a dysregulation of the PI3K/AkT/mTOR signaling cascade by both LAN and EVR, each acting at a different level and both mechanisms leading to halting of the disease.

mTOR is a serine/threonine kinase and can form two protein complexes: mTORC1 (*mTOR, Rptor*), which is inhibited EVR, and mTORC2 (*mTOR, Rictor*). Ribosomal protein S6 kinase (*S6K*) is the direct downstream target of mTORC1 and regulates the downstream translational initiation machinery to control cell growth, proliferation and autophagy. mTORC 2 controls actin cytoskeleton and resistance to apoptosis. In animals treated with EVR, we observed an increased *mTOR* gene expression which is probably due to the prolonged administration of EVR in an attempt of the cell to compensate for the inhibitory effects of EVR on *mTOR*. *S6*-protein, downstream of mTOR, was less phosphorylated in EVR-treated animals compared to control PCK rats. Renken and colleagues investigated sirolimus in PCK rats, the time-dependent effect was assessed by using different groups sacrificed at different time points. No longitudinal follow-up of LV in the same animal was performed their study. They could only observe subtle effects of sirolimus on mTOR-specific S6 kinase in the liver[26]. The large inter- and intra-litter variability of hepatic disease progression may explain why they in their study could not observe a beneficial effect on liver disease progression. Further studies on the molecular mechanisms involved in PCLD in (pre)clinical models are needed.

In conclusion, our method of LV measurement with MRI is shown to be highly sensitive and allowed us to detect accurately changes in time in a non-invasive way. Long-term everolimus treatment halts liver cyst

growth and reduces the development of fibrosis in this rat model. Our observations support the rational to explore further everolimus for the prevention of the development of symptomatic liver disease, such as in patients with ADPKD after kidney transplantation who are in need of an immunosuppressive drug.

COMMENTS

Background

The polycystic liver diseases (PCLD) represent a group of genetic disorders, in which cysts occur in the liver (ADPLD), or occur as well in the liver as in the kidneys (ADPKD). In PCLD, the liver becomes polycystic at a late stage, *e.g.*, in ADPKD patients, the prevalence is 85%, and 94% in subgroups of age (resp. 25 to 34, and 35 to 46 year). Most of the patients with PCLD are asymptomatic; however, in a subpopulation of 1%-3%, expansion of liver cysts cause invalidating abdominal symptoms furthermore, symptomatic ADPLD patients are mainly females. The most common complication in patients with PCLD is extensive hepatomegaly, which may lead to malnutrition and can be lethal and cyst-related complications include hemorrhage, infections, and rupture. There is no medical treatment approved for PCLD and to date, the only definitive treatment in those patients with large liver volumes (LV) is liver transplantation (LT).

Research frontiers

Octreotides and lanreotides have been shown to reduce LV in patients with PCLD, beneficial effects of mTOR-inhibition in patients is still a matter of debate. This was investigated in these experiments in the PCK-rat model of PCLD as preclinical evaluation. The authors designed the study representative for the hospital situation and started treatment at the moment of marked hepatomegaly (representing the symptomatic female patient). They developed and validated a MRI-based method to determine LV that can be used for longitudinal follow-up of disease progression and supported the observations with molecular analysis.

Innovations and breakthroughs

The authors developed a unique MRI-based method that was shown to be sensitive and statistically reliable. Repeat measurements allowed for monitoring individual responses and reducing the number of animals required for this type of study. This method is a great step forward for it allows preclinical testing of drugs for PCLD under controlled conditions. Everolimus looks beneficial to reduce the cyst volume in PCLD with a secondary benefit on fibrosis.

Applications

The imaging technique that they describe can be used to study drugs or drug combinations in preclinical setting in the representative animal model of PCK rats that for logistic reasons (number of symptomatic patients, slow progress of the disease, inter-individual variation of progression) is difficult to organize in patients. Ultimately, this approach can lead to a reduction of patients that require a transplantation. Polycistic liver and kidney diseases are closely related. Kidney transplantation is a frequent treatment for autosomal dominant polycystic kidney disease (ADPKD, autosomal dominant PKD or adult-onset PKD), and this study gives arguments to use everolimus in patients that need immunosuppression as it can have beneficial effects for the livers at risk in ADPKD patients and complications due to progressive fibrosis.

Peer-review

In this study, authors have shown that everolimus halts hepatic cystogenesis in a rodent model of PCLD. They have developed a MRI-based method for accurate determination of LV and investigated that everolimus halted cyst growth comparable to lanreotide and reduced the development of fibrosis, mTOR-inhibition should be further explored in PCLD patients especially those that need immunosuppression. The study has been well performed.

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P- Reviewer: Dang SS **S- Editor**: Gong ZM **L- Editor**: A
E- Editor: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5508-5518

DOI: 10.3748/wjg.v23.i30.5508

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

ORIGINAL ARTICLE

Basic Study

MicroRNA profile in neosquamous esophageal mucosa following ablation of Barrett's esophagus

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Author contributions: Watson DI, Bright T, Lord RV and Hussey DJ designed and coordinated the research; Watson DI, Lord RV and Hussey DJ obtained the funding that supported this research; Watson DI and Bright T collected patient samples; Sreedharan L, Ansar A, Wang T and Kist J prepared the tissue samples and performed the molecular studies; Sreedharan L, Mayne GC and Hussey DJ analyzed the data; Astill DS performed the histopathology; Sreedharan L, Mayne GC and Hussey DJ wrote the first draft of the paper; all authors contributed to revision of the manuscript in its final version.

Supported by National Health and Medical Research Council, Australia, No. APP1008337.

Institutional review board statement: This study was reviewed and approved by the Southern Adelaide Clinical Human Research Ethics Committee.

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

Data sharing statement: Datasets have been made available on the Gene Expression Omnibus (GEO) website, Accession number GSE94854. The data can be downloaded from https://www.ncbi.nlm.nih.gov/geo/info/download.html.

Manuscript source: Invited manuscript

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Received: February 27, 2017
Peer-review started: February 28, 2017
First decision: April 16, 2017
Revised: May 18, 2017
Accepted: July 4, 2017
Article in press: July 4, 2017
Published online: August 14, 2017

Abstract

AIM

To investigate the microRNA expression profile in esophageal neosquamous epithelium from patients who had undergone ablation of Barrett's esophagus.

METHODS

High throughput screening using TaqMan® Array Human MicroRNA quantitative PCR was used to determine expression levels of 754 microRNAs in distal esophageal mucosa (1 cm above the gastro-esophageal junction) from 16 patients who had undergone ablation of non-dysplastic Barrett's esophagus using argon plasma coagulation *vs* pretreatment mucosa, post-treatment proximal normal non-treated esophageal mucosa, and esophageal mucosal biopsies from 10

controls without Barrett's esophagus. Biopsies of squamous mucosa were also taken from 5 cm above the pre-ablation squamo-columnar junction. Predicted mRNA target pathway analysis was used to investigate the functional involvement of differentially expressed microRNAs.

RESULTS

Forty-four microRNAs were differentially expressed between control squamous mucosa *vs* post-ablation neosquamous mucosa. Nineteen microRNAs were differentially expressed between post-ablation neosquamous and post-ablation squamous mucosa obtained from the more proximal non-treated esophageal segment. Twelve microRNAs were differentially expressed in both neosquamous *vs* matched proximal squamous mucosa and neosquamous *vs* squamous mucosa from healthy patients. Nine microRNAs (miR-424-5p, miR-127-3p, miR-98-5p, miR-187-3p, miR-495-3p, miR-34c-5p, miR-223-5p, miR-539-5p, miR-376a-3p, miR-409-3p) were expressed at higher levels in post-ablation neosquamous mucosa than in matched proximal squamous and healthy squamous mucosa. These microRNAs were also more highly expressed in Barrett's esophagus mucosa than matched proximal squamous and squamous mucosa from controls. Target prediction and pathway analysis suggests that these microRNAs may be involved in the regulation of cell survival signalling pathways. Three microRNAs (miR-187-3p, miR-135b-5p and miR-31-5p) were expressed at higher levels in post-ablation neosquamous mucosa than in matched proximal squamous and healthy squamous mucosa. These miRNAs were expressed at similar levels in pre-ablation Barrett's esophagus mucosa, matched proximal squamous and squamous mucosa from controls. Target prediction and pathway analysis suggests that these microRNAs may be involved in regulating the expression of proteins that contribute to barrier function.

CONCLUSION

Neosquamous mucosa arising after ablation of Barrett's esophagus expresses microRNAs that may contribute to decreased barrier function and microRNAs that may be involved in the regulation of survival signaling pathways.

Key words: Neosquamous; Barrett's esophagus; Ablation

Core tip: We report that the microRNA profile of esophageal neosquamous mucosa developing after ablation of Barrett's esophagus is different to normal squamous epithelium, and that the differentially expressed microRNAs in neosquamous mucosa may regulate survival signalling pathways and contribute to decreased barrier function in the esophagus.

Sreedharan L, Mayne GC, Watson DI, Bright T, Lord RV, Ansar A, Wang T, Kist J, Astill DS, Hussey DJ. MicroRNA profile in neosquamous esophageal mucosa following ablation of Barrett's esophagus. *World J Gastroenterol* 2017; 23(30): 5508-5518 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5508.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5508

INTRODUCTION

The incidence of esophageal adenocarcinoma has increased rapidly in the western world over recent decades, with overall 5-year survival rates of approximately 15%[1]. A strategy to improve survival outcome is early detection of cancer, or detection at the pre-malignant stage - high grade dysplasia. Barrett's esophagus is the precursor to adenocarcinoma[2], and results from a metaplastic change of normal esophageal squamous epithelium to columnar epithelium with intestinal differentiation[3,4]. This is a consequence of chronic gastro-esophageal reflux, and it can be identified in 1%-2% of individuals aged over 60[4]. Barrett's esophagus progresses to cancer in a sequential manner through low and then high grade dysplasia[2]. The risk of progression of non-dysplastic Barrett's esophagus to adenocarcinoma has been reported to be 0.2%-0.5% per patient year for patients enrolled in surveillance programs[1].

Endoscopic surveillance remains the mainstay of cancer prevention in individuals with Barrett's esophagus, and definitive management by surgery or endoscopy is reserved for individuals who develop high grade dysplasia or cancer[5]. Several endoscopic treatments are widely used for the treatment of high grade dysplasia or early cancer in Barrett's esophagus, including radiofrequency ablation, argon plasma coagulation, and endoscopic mucosal resection.

Generally, endoscopic therapy for Barrett's esophagus aims to completely eradicate any columnar mucosa, although persistent genomic alterations at tumour suppressor loci have been found after ablation[6]. Even though endoscopic removal of Barrett's esophagus by ablative therapies is possible in the majority of patients[7], there is still a risk of recurrence and progression to adenocarcinoma following complete eradication of Barrett's esophagus. As a result endoscopically treated patients are maintained under surveillance[4].

There have also been concerns about the risk of recurrence associated with residual sub-squamous glandular tissue which is not visible with a white light endoscope. However, Basu *et al*[8] reported that sub-squamous glandular tissue was not associated with recurrence of Barrett's esophagus in patients with effective acid suppression after argon plasma

coagulation ablation, and newer ablation modalities such as radio frequency ablation appear to have largely eliminated this problem[9], due to increased control of depth and uniformity of tissue ablation[10]. There have been several reports of patients progressing to cancer in whom Barrett's esophagus has been completely eradicated[11-13], and this has brought into question the "normality" of the regenerated neosquamous epithelium. In a previous study we observed that the expression levels of cytokeratins CK-8 and CK-14, and microRNA-205, are similar in post-ablation neosquamous epithelium and more proximal normal squamous epithelium from patients with Barrett's esophagus. However, microRNA-143 expression, which is elevated in Barrett's esophagus[14], was elevated in post-ablation neosquamous mucosa and in squamous mucosa above the metaplastic segment compared to squamous epithelium from healthy patients, suggesting that the regenerated neosquamous mucosa might not be "normal"[15].

MicroRNAs are short (about 22 nucleotides) non-coding RNA molecules that regulate gene expression. Because a single microRNA can target several mRNAs, dysregulated microRNA expression can impact on key biological pathways and contribute to cancer development[16]. Dysregulated microRNA expression along the squamous - Barrett's - dysplasia - adenocarcinoma pathway has been reported by several groups[3,14,17-19]. In the current study we profiled global microRNA expression in esophageal mucosa before and after ablation of Barrett's esophagus using Argon plasma coagulation. Our aim was to further investigate differences in microRNA expression between neosquamous and normal squamous mucosa, and to investigate how these differences might contribute to altered biology using predicted targets and pathway analysis.

MATERIALS AND METHODS

The methods used for the tissue collection and processing are described in our previous study[15], and reproduced here for completion.

Tissue collection

Esophageal mucosal tissue was collected before and after Argon plasma coagulation ablation of Barrett's esophagus from 16 individuals of median age 54.2 years (range 28.9-68.1) who were enrolled in the treatment arms of previously reported randomised controlled trials of Barrett's esophagus ablation *vs* endoscopic surveillance[20]. Barrett's esophagus was defined as columnar epithelium in the distal esophagus with histological confirmation of the presence of intestinal metaplasia. All patients were free of reflux symptoms following treatment of gastro-esophageal reflux, by either high dose proton pump inhibitors (n = 8) or a laparoscopic fundoplication (n = 8); before enrolment in the trial, at pre and post-treatment sample collection, and at Barrett's esophagus ablation.

All patients underwent baseline endoscopy and biopsies from the distal esophageal mucosa were collected as described below. Biopsies were assessed by standard histopathological techniques. The presence of intestinal metaplasia and the absence of dysplasia within the Barrett's esophagus segment were confirmed in all patients. The pre-ablation length of Barrett's esophagus ranged from 1-10 cm in length (median 3 cm). Patients underwent endoscopic Argon plasma coagulation ablation following baseline endoscopy. The details of the ablation protocol have been described in detail previously[20]. Of the patients contributing tissues to the current study, complete ablation of the Barrett's esophagus was achieved in 13 of 16 (82%). In the other 3, 95%, 99% and 95% ablation was achieved. Patient-matched post ablation neosquamous and proximal squamous samples were included in the study.

Biopsy collection

Four quadrant esophageal biopsies were taken commencing from 1 cm above the gastro-esophageal junction and then every 2 cm proximally for the length of the Barrett's segment and sent for histopathology. An additional three biopsies were collected for research purposes from each sampled level of the Barrett's esophagus, and stored in RNAlater® (Ambion, Austin, Texas, United States) as per the manufacturer's protocol.

Repeat endoscopy was performed at a median of 6 wk (inter quartile range 4.96-6.5 wk) after the last ablation treatment and biopsies were collected from the post-ablation neosquamous esophageal mucosa using the same biopsy collection protocol. Additional biopsies were collected 5 cm above the proximal margin of the pre-ablation Barrett's mucosa, and the corresponding site post-ablation for use as patient-matched non-regenerated squamous esophageal mucosa.

Biopsies collected from patients who had undergone Barrett's esophagus ablation were selected for analysis from the following sites: (1) pre-ablation Barrett's esophagus mucosa (columnar mucosa with intestinal metaplasia), 1 cm above the gastro-esophageal junction; (2) post-ablation neosquamous mucosa, 1 cm above the gastro-esophageal junction; and (3) post-ablation squamous mucosa, 5 cm above the level of the pre-ablation squamo-columnar junction.

Endoscopic esophageal mucosal biopsies were also collected at endoscopy from fourteen control individuals of median age 51.9 (range 24.1-71.0) with no known esophageal disease. These biopsies were taken from the distal esophagus, at an equivalent distance from the gastro-esophageal junction to the neosquamous mucosal biopsies, to allow direct comparison of the biopsies from normal squamous mucosa from the

control patients to the biopsies of neosquamous mucosa from the patients who underwent ablation. The inclusion criteria for the control patients were: (1) no reflux symptoms; (2) endoscopy was not undertaken for the investigation of reflux; (3) no macroscopic esophagitis seen at endoscopy; (4) gastro-esophageal junction closed when viewed from within the esophagus; and (5) gastro-esophageal junction snug around the endoscope when viewed with the retroflexed endoscope.

Biopsy processing and RNA extraction

All biopsy samples for the study were immediately stored in RNAlater® (Ambion, Austin, Texas, United States) as per the manufacturer's protocol at -20 ℃ until required. When required, the samples were thawed and RNAlater removed. Twenty-five precent of each tissue sample was fixed in formalin and embedded in paraffin for histopathology to confirm that the sample contained the required epithelium. This protocol has been described in detail previously[21]. There were no buried sub-squamous columnar glands detected by histopathology in any of the biopsies of neosquamous mucosa used in this study. The remaining tissue was used for gene expression analysis. RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, United States), and RNA concentration was determined using a Biophotometer (Eppendorf AG, Hamburg, Germany).

Taqman® open array® Micro-RNA profiling

TaqMan® Array Human MicroRNA Card Set (A and B) v3.0 was used to profile the expression of 754 microRNAs[22]. The extracted RNA was reverse transcribed using Megaplex™ RT Primers Pool A and B. Each reverse transcription reaction had a final volume of 7.5 μL, and contained 45 ng of total RNA in 3 μL, and 4.5 μL of RT reaction mix containing reverse transcriptase, Megaplex™ RT Primers Pool A and B, and other reverse transcription agents. The recommended RT thermal cycling conditions were used: (16 ℃, 2 min; 42 ℃, 1 min; 50 ℃, 1 s for 40 cycles); 85 ℃, 5 min; 4 ℃ hold. 2.5 μL of RT product (cDNA) was added to 22.5 μL of PreAmp Reaction mix, containing Megaplex™ RT Primers Pool A or B and TaqMan® PreAmp Master Mix to increase the quantity of cDNA prior to PCR on the Taqman® Open Array® Micro-RNA Panels. The final volume (25 μL) of preamplification reaction mix underwent the following thermo cycling conditions: 95 ℃ 10 min; 55 ℃, 2 min; 72 ℃, 2 min; (95 ℃, 15 s, 60 ℃, 4 min for 12 cycles), 99.9 ℃, 10 min; 4 ℃ hold. Four microlitres of each preamplified product was diluted in ultrapure water (156 μL) to give a final dilution of 1:40 as per recommended protocol. For the real-time PCR 22.5 μL of Taqman® Open Array® Real-Time PCR Master Mix was added to 22.5 μL of preamplification product to give a total volume of 45 μL. Five microlitres of each PCR Reaction mix was added to 8 wells on an OpenArray® 384-Well-sample plate. The Taqman® Open Array® Micro-RNA Panel was loaded with the samples from the 384-Well-sample plate using the standard AccuFill™ method. Open Array® Real-Time qPCR was performed on the loaded Taqman® Open Array® Micro-RNA Panel using an Open Array® Real-Time PCR instrument and recommended software.

Analysis of microRNA expression data

Raw fluorescence data was exported from the OpenArray® Real-Time qPCR Analysis Software (BioTroveTM, version 1.0.4) to a comma delimited text file. A Ct (cycle threshold) value was determined for each individual qPCR assay by using the statistical software R (version 3.0.2) to fit a 3-parameter logistic curve, assuming an amplification efficiency of 2, to the raw fluorescence data of each microRNA, and the Ct of each qPCR was determined using the second derivative maximum of the fitted logistic curve. PCR reactions that did not amplify were assigned a Ct value of 40.

To investigate whether any of the samples had low quality data we used the "detector profiling across samples" module in the RealTime PCR Statminer® software analysis program (v4.5, Integromics) to examine the correlations of Ct values between samples from the same epithelial tissue type across all of the amplified microRNAs. Samples that had multiple outliers were excluded from further analysis.

For normalisation of the OpenArray® microRNA expression data we selected 14 microRNAs using the following criteria: (1) they were expressed in all samples and at high levels (median Ct < 30); (2) they were not statistically different in epithelial tissue type comparisons (Welch's *t*-test, $P > 0.1$); and (3) they were the least variable miRNAs (coefficient of variation < 1.0 for relative levels in each epithelial tissue type). The values for these selection criteria for each of the 14 microRNAs used for normalisation, plus mature nucleic acid sequences and Accession numbers, are presented in Supplementary Table 1.

The relative levels of the microRNAs were determined using the formula $2^{(40-Ct)}$, and were normalized using the geometric mean of the relative levels of the 14 House Keeping Genes. The data was pre-filtered using the following criteria to include microRNAs that were more likely to be informative: (1) each microRNA had to have at least 50% of samples amplified in one of the comparison groups; and (2) the differential expression between groups had to be greater than 1.4 fold. Mann Whitney *U* tests were then used to discover differentially expressed microRNAs in control squamous mucosa *vs* post-ablation neosquamous mucosa, and in post-ablation squamous mucosa *vs* post-ablation neosquamous mucosa. False discovery rates (the proportions of false positives) were estimated for each epithelial tissue type comparison. MicroRNAs that had $P < 0.05$ in both of these epithelial tissue type comparisons were termed "overlapping miRNAs".

Subset analyses were subsequently performed for these overlapping microRNAs in 2 sub-groups: (1) patients who were treated either medically or surgically for reflux; and (2) patients in whom complete ablation was achieved *vs* all patients. This was done by averaging the differential expression and the Mann Whitney *U* test *P* values in each patient subgroup for the overlapping microRNAs. Differences between the groups in (1) differential expression; and (2) Mann Whitney *U* test *P* values were tested using Welch's *t*-test. The statistical methods of this study were reviewed by Professor Richard Woodman from Flinders University.

The overlapping miRNAs were further investigated to compare the direction of differential expression of these microRNAs in post-ablation neosquamous mucosa *vs* control squamous mucosa and post-ablation squamous mucosa, to the direction of differential expression in Barrett's esophagus mucosa *vs* control squamous mucosa and post-ablation squamous mucosa. The potential roles of the overlapping miRNAs in regulating cellular processes were investigated using biological pathway enrichment analysis (described in next section).

Biological pathway enrichment analysis of overlapping microRNAs

To identify highly predicted mRNA targets of the differentially expressed microRNAs in neosquamous mucosa, we used the Predicted Target Module of miRWalk v2[23] (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). To generate the putative target genes list we used a minimum seed length of 7 and/or *P* value < 0.05, from position 1 of the 3′ UTR, and included extra databases: RNA22 (https://cm.jefferson.edu/rna22/), miRanda (http://www.microrna.org/microrna/home.do) and Targetscan (http://www.targetscan.org/vert_71/). The predicted lists for each microRNA were then screened to identify mRNAs that were predicted to be the targets of at least two different microRNAs.

To identify pathways containing a statistically significant number of predicted targets, we used a publicly available, manually curated signalling pathway database[24] (http://www.innatedb.com/redirect.do?go=batchPw). The target list was subjected to a Pathway Enrichment Analysis which groups target genes according to function, and identifies further components and associated networks. The target list (in REFSeq ID format) was analysed using InnateDB, which identifies statistically enriched pathways by testing for over-representation using the Hypergeometric distribution (by default; other distributions are available), and by using the Benjamini Hochberg correction for multiple tests (by default). InnateDB uses multiple curated databases for the pathway analysis: Reactome (http://www.reactome.org/), KEGG (http://www.genome.jp/kegg/), PID Biocarta and PID NCI (http://www.home.ndexbio.org/), NetPath (http://www.netpath.org/), INOH (only available within InnateDB).

RESULTS

Post-ablation neosquamous vs control squamous mucosa comparison

Forty-four microRNAs were differentially expressed at *P* < 0.05 between control squamous mucosa and post-ablation neosquamous mucosa (Supplementary Table 2). Thirty-three of these microRNAs had higher expression in post-ablation neosquamous mucosa *vs* control squamous mucosa, and 25 of these had a fold difference greater than 2. There were 11 microRNAs that had lower expression in post-ablation neosquamous mucosa *vs* control squamous mucosa, although only 2 of these were expressed at levels of 50% or less.

Post-ablation neosquamous vs post-ablation squamous mucosa comparison

Nineteen microRNAs were differentially expressed at *P* < 0.05 between post-ablation neosquamous and post-ablation squamous mucosa (Supplementary Table 3). Fourteen microRNAs had higher expression and 5 microRNAs had lower expression in post-ablation neosquamous mucosa compared with post-ablation squamous mucosa.

MicroRNAs that were different in both control squamous vs post-ablation neosquamous mucosa and post-ablation neosquamous vs post-ablation squamous mucosa

Due to the large number of microRNAs that were assayed it is possible that some differentially expressed microRNAs occurred by chance alone and are thus false positives. We therefore estimated the false discovery rate (FDR) in each epithelial tissue type comparison: in control squamous *vs* post-ablation neosquamous mucosa the FDR was 11%, and in the post-ablation squamous *vs* post-ablation neosquamous mucosa the FDR was 19%. The post-ablation squamous *vs* post-ablation neosquamous comparison could also identify microRNAs associated with differential expression along the length of the esophagus[25]. In order to address these issues we investigated whether there were microRNAs that were differentially expressed in both epitheilial tissue type comparisons. We reasoned that because the control squamous mucosa samples were obtained from different patients to the post-ablation squamous mucosa, any differentially expressed microRNAs found in both mucosal comparisons were much less likely to be due to chance alone. This approach identified 12 microRNAs that were present in both control squamous *vs* post-ablation neosquamous mucosa and the post-ablation squamous *vs* post-ablation neosquamous mucosa groups (Table 1). Scatter plots for the 12 overlapping microRNAs are in Supplementary Figure 2. OpenArray assay identifiers, miRBase names and accession numbers, and mature nucleotide sequences for these overlapping microRNAs

Table 1 Fold differences in gene expression and Mann Whitney *U* test *P* values for the microRNAs that were differentially expressed in both post-NS *vs* control-S and post-NS *vs* post-S mucosa comparisons

Mature miRNA	Post-NS/control-S	*P* value	Post-NS/ Post-S	*P* value	Higher in pre-BE *vs* post-NS
miR-424-5p	485.2	0.00002	233.7	0.00053	Yes
miR-135b-5p	2.0	0.00071	1.7	0.00363	No
miR-376c-3p	6.4	0.00145	3.1	0.02673	Yes
miR-135a-5p	3.0	0.00224	2.5	0.00821	Yes
miR-187-3p	208.1	0.00414	2.7	0.01196	No
miR-409-3p	6.8	0.00502	4.6	0.00272	Yes
miR-214-5p	47.0	0.00502	46.1	0.02673	Yes
miR-31-5p	1.44	0.00869	1.5	0.00632	No
miR-199a-5p	396.3	0.01223	512.9	0.04478	Yes
miR-223-5p	230.9	0.02306	204.5	0.01350	Yes
miR-127-3p	4.8	0.02675	3.8	0.03305	Yes
miR-136-3p	7.2	0.02675	201.2	0.03305	Yes

Table 2 Predicted molecular pathways of the mRNA targets of differentially expressed microRNAs that are increased in neosquamous but not Barrett's esophagus mucosa

Pathway name	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway *P* value	Pathway *P* value (corrected)
Ion transport by P-type ATPases	5	43	1.57E-05	0.005
Transmembrane transport of small molecules	14	606	1.31E-04	0.022
Ion channel transport	7	169	2.56E-04	0.028
Calcium signaling pathway	7	183	4.15E-04	0.035

Predicted molecular pathways using miRWalk to generate predicted mRNA targets of the microRNAs differentially expressed in both Neosquamous *vs* healthy control and Neosquamous *vs* Proximal squamous comparisons, and InnateDB to identify pathways in which the mRNA targets are over-represented.

are in Supplementary Table 4. All of these microRNAs were more highly expressed in post-ablation neosquamous tissues, and 10 of the 12 overlapping microRNAs had similar fold differences in the two groups. In both comparisons miR-424-5p was the most significantly differentially expressed microRNA (Table 1). We further investigated these overlapping miRNAs in subsets of the data (patients with complete ablation *vs* all patients, and patients who were medically treated *vs* surgically treated for reflux) and did not find significant differences in differential expression between these subset groups (Supplementary Tables 7 and 8).

The 12 overlapping microRNAs were further investigated to determine their levels of expression in pre-ablation Barrett's esophagus mucosa. The expression levels of 9 of the 12 overlapping microRNAs were higher in Barrett's esophagus mucosa than in the squamous mucosa, and for all of these 9 microRNAs their expression in post-ablation neosquamous mucosa was in the same direction (*i.e.,* higher) as in the Barrett's esophagus mucosa (Table 1; Figure 1A for a representative example). For the remaining 3 microRNAs the levels in the Barrett's esophagus mucosa were not different to the non-neosquamous mucosa (Figure 1B for a representative example).

Target prediction and pathway analysis of the overlapping microRNAs

For the 3 overlapping microRNAs that were found not to be increased in Barrrett's esophagus mucosa relative to non-neosquamous mucosa mirWalk predicted 1566 mRNA targets, and 163 mRNAs with 2 or more microRNAs targeting them (Supplementary Table 5). Pathway analysis using InnateDB indicated that the predicted target mRNAs are involved in active membrane transport and in calcium signalling (Table 2). For the 9 microRNAs that are increased in both neosquamous and Barrett's esophagus mucosa relative to squamous mucosa mirWalk predicted 3297 mRNA targets, and 839 mRNAs with 2 or more microRNAs targeting them (Supplementary Table 6). Pathway analysis using InnateDB indicated that the predicted target mRNAs are involved in hemostasis and in cell survival pathways (Table 3).

DISCUSSION

Over the last decade, endoscopic treatment of high grade dysplasia and early cancer arising in Barrett's esophagus has largely superseded surgical resection, because of the perception of reduced morbidity, and the lower risk of lymph node metastases when cancer stage is limited to no worse than stage T1a[26,27]. Consensus guidelines for endoscopic therapy suggest that complete eradication of all Barrett's esophagus mucosa is required to eliminate the risk of metachronous and covert synchronous neoplasia[28,29]. However, increasing evidence suggests that complete

Table 3 Predicted molecular pathways of the mRNA targets of differentially expressed microRNAs that are increased in both neosquamous and Barrett's esophagus mucosa

Pathway name	Pathway uploaded gene count	Genes in InnateDB for this entity	Pathway *P* value	Pathway *P* value (corrected)
JAK STAT pathway and regulation	28	273	5.41E-06	0.005
Hemostasis	41	508	1.68E-05	0.005
Regulation of bad phosphorylation	7	23	2.36E-05	0.006

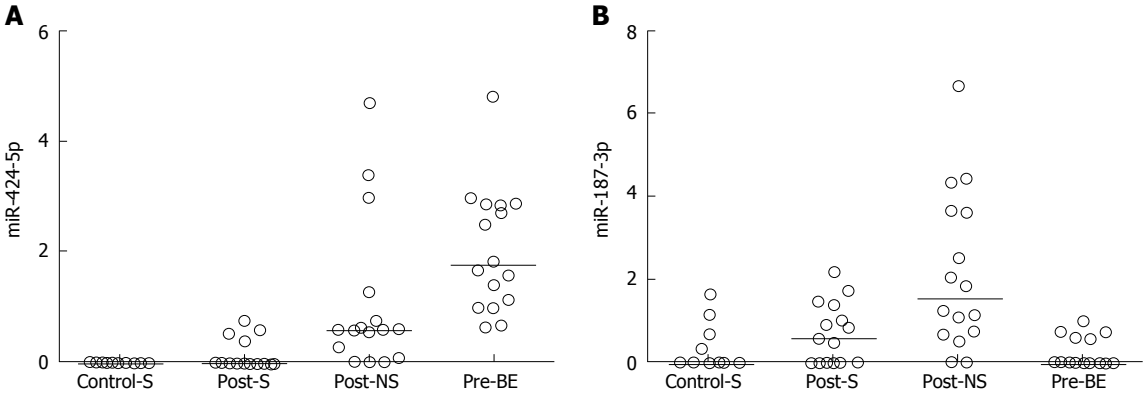

Figure 1 Normalised relative expression levels in control squamous mucosa (control-S), post-ablation squamous mucosa (post-S), post-ablation neosquamous mucosa (post-NS) and pre-ablation Barrett's esophagus mucosa (pre-BE) of representative microRNAs, miR-424-5p (A) and miR-187-3p (B). Horizontal bars are medians.

eradication of Barrett's esophagus might not eliminate the risk of cancer in some patients. For example, Templeton *et al*[11] (2014) reported three patients who progressed to invasive adenocarcinoma despite prior complete eradication of Barrett's esophagus using endoscopic therapy. Even after re-treatment and complete endoscopic eradication of the post-ablation recurrences of Barrett's esophagus, Guthikonda *et al*[12] (2016) reported a progression rate to invasive cancer of 2.1% per year, and predicted that 5.1% (Kaplan Meier model estimate; 95%CI: 0.0-11.3) of re-treated patients would experience invasive cancer progression by 5 years after complete eradication of Barrett's esophagus[12]. In a meta-analysis of 21 radiofrequency ablation studies that reported 603 cases of Barrett's esophagus recurrence from 3186 patients, pooled incidence ratios (IR's) of recurrent Barrett's esophagus, dysplastic Barrett's esophagus, and HGD/EAC were 9.5% (95%CI: 6.7-12.3), 2.0% (95%CI: 1.3-2.7), and 1.2% (95%CI: 0.8-1.6) per patient-year, respectively[13]. Ongoing endoscopic surveillance has therefore been recommended after eradication of Barrett's esophagus to monitor for recurrence and disease progression.

It has been suggested that residual sub-squamous glandular tissue after ablation may contribute to the progression of Barrett's esophagus to high grade dysplasia and adenocarcinoma[30,31]. For example, it has been reported that after argon plasma coagulation ablation buried glandular tissue underneath neosquamous mucosa had higher levels of cancer associated biomarkers (Ki67, COX-2, BCL-2) than normal esophageal epithelium[32]. However, Basu *et al*[8] (2002) reported that the presence of buried Barrett's glands was not associated with recurrence in patients with effective acid suppression after argon plasma coagulation ablation. Furthermore, neosquamous epithelium has been reported not to contain genetic abnormalities after radio frequency ablation in patients who had pre-ablation Barrett's esophagus containing early cancer or high-grade dysplasia[9].

In an earlier study we investigated neo-squamous mucosa in patients who had undergone argon plasma coagulation ablation of non-dysplastic Barrett's esophagus, and observed that expression levels of miR-143 were elevated in neosquamous epithelium and biopsies from squamous epithelium above the metaplastic segment, compared to squamous epithelium from controls without Barrett's esophagus[15]. miR-143 has also been shown to have increased expression in Barrett's esophagus mucosa. This study suggested that post-ablation neosquamous epithelium might not be normal, and might express persistent molecular markers consistent with the original Barrett's esophagus.

In our current study we used post-ablation mucosa from patients who did not have dysplasia or early cancer to further investigate the biology of the neosquamous epithelium, and it is unlikely that these biopsies would have contained residual dysplasia associated biomarkers[32], or be effected by DNA mutations that are commonly found in dysplastic tissues[6].

We sought to strengthen our approach by restricting the list of microRNAs to those that are differentially

expressed in both neosqamous *vs* independent squamous mucosa from control individuals, and in neosquamous *vs* paired proximal squamous mucosa comparisons. This approach should minimise the issues associated with inter-individual variation, epithelial repair, and tissue proximity to the gastro-esophageal junction as possible causes of differences in microRNA expression. This approach produced 12 microRNAs that were differentially expressed in both of the squamous mucosa comparisons.

To investigate the potential biological effects of the differential expression of these 12 microRNAs we utilized miRWalk to predict their mRNA targets, and InnateDB to assess which potential signalling pathways these mRNAs are involved in. This approach identified signalling pathways which the differentially expressed miRNAs might be regulating.

Nine microRNAs were expressed at higher levels in both neosquamous and pre-ablation Barrett's mucosa, *vs* both squamous mucosa from controls and proximal squamous mucosa collected after ablation from the ablation patients. The predicted mRNA targets of these microRNAs are involved in the JAK-STAT signalling pathway, and in the regulation of the anti-apoptotic family member, Bad. Three of the discovered microRNAs have been reported to have targets involved in hemostasis[33-35].

The JAK-STAT signalling pathway transmits information from extracellular signals directly to the cell nucleus, and results in expression of genes involved in proliferation, differentiation, apoptosis and oncogenesis. The JAK-STAT3 pathway is activated by IL-6 in Barrett' s esophagus, and this promotes survival of the metaplastic intestinal cells[36,37]. However, activated JAK-STAT has been reported to be undetectable in normal esophageal squamous mucosa[38], so the effect of altered regulation of this pathway in neosquamous mucosa is not clear.

Three microRNAs were increased in neosquamous, but not pre-ablation Barrett's esophagus mucosa, *vs* both healthy squamous and post-squamous mucosa. The predicted mRNA targets of these microRNAs are potentially involved in regulating pathways involved in transmembrane transport of small molecules, in ion channel transport, and in ion and lipid transport by P-type ATPases (which includes the calcium pump, Ca^{2+}-ATPase). Jovov *et al*[39] (2013) found that post-ablation neosquamous epithelium has decreased barrier function, measured as persistent paracellular permeability to ions and uncharged molecules[39], so these active transporter targets are likely to be involved in the regulation of barrier protection. This is an important consideration because acid reflux injury has been implicated in the development of intestinal metaplasia, and decreased barrier function may therefore contribute to recurrence in patients with uncontrolled reflux following successful ablation.

The mucosal biopsies used in this study were from patients treated with Argon Plasma Coagulation ablation. These were collected as part of clinical trials previously established in our institution and were therefore readily available to us[20]. The use of biopsies from patients treated with argon plasma coagulation ablation is a potential limitation of our study, since radiofrequency ablation is now the standard technique for ablation of Barrett's esophagus due to its ease of use and consistent depth of tissue destruction[40]. However, some patients in whom complete eradication has been achieved following radiofrequency ablation have still progressed to invasive cancer, which suggests that post radiofrequency ablation neosquamous epithelium may also not be normal. It is worth noting that the above described report of decreased barrier function in neosquamous mucosa was associated with radiofrequency ablation[39]. Future studies of neosquamous epithelium miRNA profile should therefore include other endoscopic methods such as radiofrequency ablation and endomucosal resection, and validation in independent cohorts.

The observed decrease in barrier function in neosquamous mucosa is consistent with reports of an association between recurrence after ablation and persistent reflux, and with reduced proton pump inhibitor dosing. Kahaleh *et al*[41] (2002) found that persistence of acid reflux and greater length of diseased segment were the major factors associated with a recurrence after successful initial reversal with argon plasma coagulation ablation. Basu *et al*[8] (2002) reported that patients who reduced their dose of the proton pump inhibitor omeprazole to 20 mg once daily or less after argon plasma coagulation ablation had significantly greater recurrence of intestinal metaplasia[8]. Conversely, in patients with complete squamous regeneration after argon plasma coagulation ablation who took a high dose of omeprazole (40 mg three times a day) there were no relapses or evidence of dysplasia under continuous acid suppression during a median follow-up of 12 mo (range 2 to 51 mo)[42].

Three (miR-424-5p, miR-223-5p, miR-409-3p) of the microRNAs that are increased in neosquamous mucosa relative to post-squamous and control-squamous mucosa have been reported to be up-regulated in esophageal adenocarcinoma relative to Barrett's esophagus and normal squamous tissues. Wu *et al*[18] (2013) observed progressively increased expression of miR-424-5p, miR-223-5p and miR-409-3p from normal squamous epithelium to Barrett's to adenocarcinoma. MiR-223-5p and miR-409-3p have also been reported to be overexpressed in serum exosomes from patients with esophageal adenocarcinoma[43].

In conclusion, this study demonstrates that the miRNA expression profile in neosquamous mucosa, following argon plasma coagulation ablation of Barrett's esophagus, is significantly different from normal squamous mucosa. The main strength of this study is that the mucosal

biopsies were not from patients who were treated for dysplasia or cancer. Our results suggest that altered miRNA expression may contribute to the previously reported defective barrier function in neosquamous epithelium, and this may place the mucosa at increased risk of disease progression relative to normal esophageal squamous mucosa. Further research to explore the roles of miRNAs in the response to ablation of Barrett's esophagus, and the long term behaviour of neosquamous epithelium may lead to improvements in clinical management of this condition.

ACKNOWLEDGMENTS

We thank the SA Pathology anatomical pathology laboratory for assistance with preparation of histopathology slides.

COMMENTS

Background

Overall 5 year survival of patients with esophageal adenocarcinoma is around 15%, but survival can be improved *via* early detection using endoscopic surveillance of patients with Barrett's esophagus, and endoscopic ablative therapy for the treatment of early stage disease.

Research frontiers

Some patients still experience disease progression after complete ablation of Barrett's esophagus, which suggests that the neosquamous mucosa formed after ablation may be at increased risk of disease progression. Improved understanding of the biological properties of post-ablation neosquamous mucosa might improve the clinical management of patients who undergo ablative therapy.

Innovations and breakthroughs

Previous studies have investigated whether post ablation mucosa has genetic alterations in, or increased expression of cancer associated genes. Neither of these approaches directly addresses whether the non-cancer associated biology of neosquamous mucosa is different. Previous studies have reported reduced barrier function in neosquamous mucosa, and this may have implications for clinical management.

Applications

Altered microRNA expression in neosquamous mucosa might result in reduced barrier function, thereby placing the mucosa at increased susceptibility to reflux induced disease. MicroRNAs might therefore have the potential to be developed into a biomarker with clinical utility to improve the management of patients who have been treated endoscopically for early stage disease.

Terminology

Post-ablation neosquamous mucosa: post-treatment regenerated esophageal squamous mucosa that was Barrett's intestinal metaplasia prior to treatment, with biopsies taken 1 cm above the gastro-esophageal junction. Post-ablation squamous mucosa: post-treatment proximal normal non-treated esophageal mucosa, with biopsies collected 5 cm above the proximal margin of the pre-ablation Barrett's mucosa. Healthy squamous mucosa: squamous mucosa from patients without esophageal disease, with biopsies taken from the same level relative to the gastro-esophageal junction as the post-ablation neosquamous mucosa.

Peer-review

This manuscript evaluates a topic of real interest. The analysis of the subject has been done in an appropriate way. The background of the problem was evaluated in a comprehensive way, the hypothesis was clearly stated, and the materials, methods and results are presented in an understandable way (I will add minor comments).

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P- Reviewer: Durand L, Garcia-Olmo D **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Li D

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5519-5529

DOI: 10.3748/wjg.v23.i30.5519

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

ORIGINAL ARTICLE

Basic Study

Expression of Interleukin-26 is upregulated in inflammatory bowel disease

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Institutional review board statement: This study was reviewed and approved by the local ethics committee of the Shiga University of Medical Science (Permit number: 27-27).

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

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: April 7, 2017
Peer-review started: April 10, 2017
First decision: June 8, 2017
Revised: June 26, 2017
Accepted: July 12, 2017
Article in press: July 12, 2017
Published online: August 14, 2017

Abstract

AIM

To investigate interleukin (IL)-26 expression in the inflamed mucosa of patients with inflammatory bowel disease (IBD) and the function of IL-26.

METHODS

Human colonic subepithelial myofibroblasts (SEMFs) were isolated from colon tissue surgically resected. The expression of IL-26 protein and its receptor complex was analyzed by immunohistochemistry. The gene expression induced by IL-26 was evaluated by real-time polymerase chain reaction. Intracellular signaling pathways were evaluated by immunoblotting and specific small interfering (si) RNA transfection.

RESULTS

The mRNA and protein expression of IL-26 were significantly enhanced in the inflamed mucosa of patients with IBD. IL-26 receptor complex was expressed in colonic SEMFs *in vivo* and *in vitro*. IL-26 stimulated the mRNA expression of IL-6 and IL-8 in colonic SEMFs. The inhibitors of mitogen-activated protein kinases and phosphoinositide 3-kinase, and siRNAs for signal transducers and activator of transcription 1/3, nuclear factor-kappa B and activator protein-1 significantly reduced the mRNA expression of IL-6 and IL-8 induced by IL-26.

CONCLUSION
These results suggest that IL-26 plays a role in the pathophysiology of IBD through induction of inflammatory mediators.

Key words: Inflammatory bowel disease; Interleukin-26; Myofibroblasts

Core tip: We investigated interleukin (IL)-26 expression in the inflamed mucosa of patients with inflammatory bowel disease (IBD) and characterized the biological function of IL-26 using human colonic subepithelial myofibroblasts. To our knowledge, this is the first report to state that IL-26 activates STAT1/3 and leads to the induction of IL-6 and IL-8 expression in non-transformed cells derived from human colon. We suggest that IL-26 plays a role in the pathophysiology of IBD through the induction of inflammatory mediators.

Fujii M, Nishida A, Imaeda H, Ohno M, Nishino K, Sakai S, Inatomi O, Bamba S, Kawahara M, Shimizu T, Andoh A. Expression of Interleukin-26 is upregulated in inflammatory bowel disease. *World J Gastroenterol* 2017; 23(30): 5519-5529 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5519.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5519

INTRODUCTION

Inflammatory bowel diseases (IBD) comprise two major phenotypes, Crohn's disease (CD) and ulcerative colitis (UC). Recent studies suggest that the chronic inflammation in IBD is mediated by uncontrolled immune responses against a subset of luminal bacteria and dietary factors[1-3]. This hypothesis is supported by the recent finding that the genes encoding innate immune responses are responsible for the susceptibility to IBD[4-6]. However, the precise etiology of IBD still remains unclear.

Interleukin (IL)-26 is a member of the IL-10 cytokine family. IL-26 was first discovered in *herpesvirus saimiri*-transformed T-cell lines by subtractive hybridization[7]. IL-26 has been reported to be co-expressed with IL-17 and IL-22 by Th17 cells[8-10], and recent studies have reported that natural killer cells[11,12], macrophages[13], and fibroblast-like cells[14,15] are sources of IL-26. A murine IL-26 homologue has not been identified[16,17], limiting the experimental opportunities to study the phenotypic consequences of IL-26 gene knockout and IL-26-mediated functions in murine models *in vivo*.

Although IL-19, IL-20, and IL-24, members of IL-10 cytokine family, are located in proximity to the IL-10 gene on human chromosome 1q32, IL-26 is located on chromosome 12q15 between the genes encoding IFN-γ and IL-22[18]. For its signaling, IL-26 requires the heterodimeric receptors composed of IL-20R1 and IL-10R2[19]. The transmembrane protein IL-20R1 has been shown to possess the specific ligand-binding site for IL-26, whereas the IL-10R2 subunit acts as an essential second chain that completes the assembly of its active receptor complex and signaling[20]. IL-22, IL-26, IL-28A/B and IL-29 also use IL-10R2 for their signaling. IL-10R2 is ubiquitously expressed in various tissues, but the expression of IL-20R1 is absent in hematopoietic cells and is restricted within non-hematopoietic cells[19,21].

Previous reports have suggested that intracellular signaling of IL-26 is mediated by the Janus kinase-signal transducer and activator of transcription (STAT) pathway[16,20]. Additionally, IL-26 has been reported to activate extracellular signal-related kinase (ERK)-1/2, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), p38 mitogen-activated protein kinase and phosphoinositide 3-kinase (PI3K) in various kinds of cells[7,16,20,22].

Human colonic subepithelial myofibroblasts (SEMFs) are located immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed against the bottom of the epithelial cells[23-25]. These cells are considered to play a role in the regulation of a number of epithelial cell functions, such as epithelial proliferation and differentiation. Isolated SEMFs retain their representative and differentiated phenotypes[23].

Recent studies have demonstrated that IL-26 is involved in the pathophysiology of chronic inflammatory disorders, such as rheumatoid arthritis[15] and chronic hepatitis C infection[26]. Concerning IBD, the pathological role of IL-26 has been reported in CD, but remains unclear in UC[16]. In addition, functional analysis of IL-26 has been studied using a transformed cell line, but there are no reports using primary culture cells. In the present study, to explore the potential role of IL-26 in IBD, we investigated the expression of IL-26 in the inflamed mucosa of UC and CD patients. Furthermore, the biological functions and the intracellular signal pathways activated by IL-26 were investigated in human colonic SEMFs.

MATERIALS AND METHODS

Reagents

All reagents and antibiotics used in this study were commercially purchased as shown in Supplementary Table 1.

Tissue samples

The diagnosis of IBD was based on conventional clinical and endoscopic criteria. Surgically-resected specimens and biopsy specimens from 49 patients with UC and 19 patients with CD were used after obtaining written informed consent. All experiments were approved by the local ethics committee of the Shiga University of Medical Science (Permit number: 27-27).

Table 1 Oligonucleotides used in this study

Gene name	Accession number		Primers
IL-26	NM_018402.1	Sense	5′-GGCAGAAATTGAGCCACTGT-3′
		Antisense	5′-TCCAGTTCACTGATGGCTTTG-3′
IL-10R2	NM_000628.4	Sense	5′-GGCTGAATTTGCAGATGAGCA-3′
		Antisense	5′-GAAGACCGAGGCCATGAGG-3′
IL-20R1	NM_014432.3	Sense	5′-TACACCCCTCAGCTCCAAGACT-3′
		Antisense	5′-GAAGGAATTACACAGCCTGCCAG-3′
IL-6	NM_000600.4	Sense	5′-GGTACATCCTCGACGGCATCT-3′
		Antisense	5′-GTGCCTCTTTGCTGCTTTCAC-3′
IL-8	NM_000584.3	Sense	5′-ATGACTTCCAAGCTGGCCGTGGCT-3′
		Antisense	5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′
β-*actin*	NM_001101.3	Sense	5′-TGACCCAGATCATGTTTGAGACCT-3′
		Antisense	5′-CCACGTCACACTTCATGATGGAG-3′
STAT1	NM_139266	Sense	5′-GGAAGGGGCCATCACATTCA-3′
		Antisense	5′-GTAGGGTTCAACCGCATGGA-3′
STAT3	NM_003150.3	Sense	5′-GGAGGAGTTGCAGCAAAAAG-3′
		Antisense	5′-GGAGGAGTTGCAGCAAAAAG-3′
c-jun	NM_002228.3	Sense	5′-CAGGTGGCACAGCTTAAACA-3′
		Antisense	5′-GTTTGCAACTGCTGCGTTAG-3′
NF-κBp65	NM_003998.3	Sense	5′-CGCATCCAGACCAACAACAA-3′
		Antisense	5′-GCATTCAGGTCGTAGTCCCC-3′

The clinical activity of IBD was determined according to the colitis activity index for UC[27] and CD activity index[28]. Normal colonic tissues were obtained from the distal part of the surgically resected sample of colon cancer (n = 3) and using colonoscopy (n = 17).

Immunohistochemistry

Immunohistochemical analyses were performed according to the method described in our previous report[29]. Briefly, goat anti-IL-26, goat anti-IL-20R1 and rabbit anti-IL-10R2 were used as the primary antibodies. After incubation with the primary antibodies, the sections were treated with HRP-labeled anti-goat or anti-rabbit antibodies. Diaminobenzidine was used as a substrate for color development.

For double-staining procedures, anti-IL-20R1 or anti-IL-10R2 antibodies were applied and incubated overnight in a humidified chamber. Subsequently, anti-α-smooth muscle actin (SMA) antibodies were applied and incubated overnight. Dylight488-labeled anti-goat IgG, Dylight549-labeled anti-mouse IgG, or Dylight549-labeled anti-mouse IgG were used as secondary antibodies. Images were obtained with a digital confocal laser scanning microscope LSM510 version 3.0 (Carl Zeiss Microscopy, Tokyo, Japan).

Culture of human colonic SEMFs

Primary cultures of colonic SEMFs were prepared according to the method reported by Mahida *et al*[30]. The cellular characteristics and culture conditions have also been described in our previous report[31]. The studies were performed on passages 3-6 of SEMFs.

Reverse transcription-polymerase chain reaction and Real-time polymerase chain reaction

The expression of mRNA in the samples was assessed by reverse transcription polymerase chain reaction (RT-PCR) and real-time PCR analysis. RT-PCR was performed according to the methods described in our previous report[32]. Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and was reverse transcribed using SuperScript II (Invitrogen). Subsequently, cDNAs were generated using SYBR Premix Ex Taq (TAKARA, Shiga, Japan), and real-time PCR was performed using a LightCycler480 System II (Roche Diagnostics, Basel, Switzerland) with specific primers for target genes. The PCR primers used in this study are shown in Table 1.

Enzyme-linked immunosorbent assay

Concentrations of IL-6 and IL-8 in cell culture supernatants were determined using ELISA kits (R&D systems, Minneapolis, MN, United States).

Silencing gene expression in human colonic SEMFs

Human colonic SEMFs were transfected with siRNA specific for STAT1, STAT3, nuclear factor (NF)-κBp65, and c-Jun according to the instructions for Lipofectamine RNAiMAX (Invitrogen). Briefly, human colonic SEMFs were cultured in complete medium without antibiotics in the presence of a mixture of an RNAi duplex and Lipofectamine RNAiMAX for 24 h, and were then stimulated with or without IL-26 for 3 h.

Nuclear and cytoplasmic protein extraction and immunoblot analysis

Nuclear proteins were extracted using the CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich, St. Louis, MO, United States). Extracted nuclear proteins were subjected to immunoblotting with rabbit anti-NF-κBp65 (C-20) antibody or mouse anti-phospho (P)-c-Jun (KM-1) antibody, followed by incubation with HRP-labeled anti-rabbit antibody or HRP-labeled anti-mouse antibody. Immunoblots were performed according to a

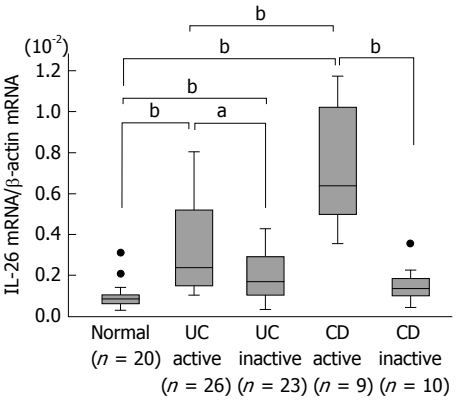

Figure 1 Expression of interleukin-26 mRNA in the inflamed mucosa of patients with inflammatory bowel disease. Total RNA was extracted from biopsied samples, and the mRNA expression of IL-26 was evaluated using real-time PCR. IL-26 mRNA expression was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for normal mucosa. Data are expressed as mean ± SE. Normal mucosa, n = 20; active UC, n = 26; inactive UC, n = 23; active CD, n = 9; inactive CD, n = 10. $^{a}P < 0.05$ UC active *vs* UC inactive, $^{b}P < 0.01$ Normal *vs* UC active, Normal *vs* UC inactive, Normal *vs* CD active, UC active *vs* CD active, CD active *vs* CD inactive. IL: Interleukin; CD: Crohn's disease; UC: Ulcerative colitis.

method previously described[33,34]. Signal detection was performed using the enhanced chemiluminescence Western blot system (GE Healthcare, Little Chalfont, United Kingdom).

Cytoplasmic protein was extracted using a lysis buffer [50 mmol/L Tris pH 8.0, 0.5% Nonidet P-40, 1 mmol/L EDTA, 150 mmol/L NaCl, 2 mmol/L Na_3VO_4, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 1 mmol/L PMSF, 10% glycerol and complete Mini Protease Inhibitor Cocktail (Roche Diagnostics)]. Extracted protein was subjected to immunoblotting with antibodies against phospho-p44/42 MAPK (ERK1/2), p38 MAPK, or SAPK/JNK, Akt, STAT1, or STAT3 followed by incubation with HRP-labeled anti-rabbit antibody or HRP-labeled anti-mouse antibody. After detection as described above, the membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Scientific Inc., Waltham, MA) and was then incubated with antibodies against total-p44/42 MAPK (ERK1/2), p38 MAPK, SAPK/JNK, Akt, STAT1, or STAT3.

Statistical analysis

Single comparisons were analyzed using the non-parametric Mann-Whitney *U* test. Differences resulting in *P* values of less than 0.05 were considered to be statistically significant. The statistical methods of this study were reviewed by a biomedical statistician from Shiga University of Medical Science.

RESULTS

IL-26 expression in IBD mucosa

The mRNA expression of IL-26 in the inflamed mucosa of IBD patients was evaluated using real-time PCR. As shown in Figure 1, IL-26 mRNA expression was faintly detected in normal mucosa. The mucosal mRNA expression of IL-26 was significantly higher in active UC patients than in the inactive UC mucosa and normal mucosa. Similar findings were also observed in the inflamed mucosa of CD patients. The average level of IL-26 mRNA expression was significantly higher in active CD mucosa than in active UC mucosa.

IL-26 protein expression was evaluated by immunohistochemical analysis. IL-26 positive cells were not detected in normal mucosa, but the number of IL-26 positive cells markedly increased in the inflamed mucosa of UC and CD patients (Figure 2A). Moreover, the number of IL-26 positive cells more increased in the inflamed mucosa of CD patients as compared to in the inflamed mucosa of UC patients (Figure 2A). This result was confirmed by the results from Figure 1. Furthermore, to identify the cellular source of IL-26, double staining was performed. As shown in Figure 2B, double staining studies indicated that IL-26-positive cells were positive for CD4 (T cell), CD56 (NCAM; NK cell), or CD68 (macrophage). These findings indicated that $CD4^+$ T cells, NK cells, and macrophages were the cellular sources of IL-26 in the inflamed mucosa of UC and CD patients.

IL-26 receptor expression in SEMFs

We looked for the presence of either IL-20R1 or IL-10R2 in the inflamed mucosa of IBD patients. It has been reported that the expression of IL-20R1 is restricted in non-hematopoietic cells, while IL-10R2 is ubiquitously expressed[19,35]. The tissue samples from the normal, the active IBD and inactive IBD were double stained with anti-α-SMA, a marker for myofibroblasts, and IL-20R1 or IL-10R2. As shown in Figure 3A, IL-20R1 was expressed in epithelial cells and in some of the other cells in the submucosa, and the cells in the subepithelial region also stained with α-SMA. IL-10R2 expression was detected in various cells including epithelial cells and leukocytes, and the cells at the subepithelial region also stained with α-SMA. The expression levels of IL-20R1 and IL-10R2 were not different between in the mucosa from the normal and in the active or inactive mucosa from IBD patients. These results suggested that human colonic SEMFs are expressing the IL-26 receptor.

We also confirmed the IL-20R1 and IL-10R2 expression in isolated human colonic SEMFs. As shown in Figure 3B, isolated human colonic SEMFs expressed IL-20R1 and IL-10R2 mRNAs.

Effects of IL-26 in SEMFs

Based on the expression of IL-26 receptor in colonic SEMFs, we examined the biological effect of IL-26 on human colonic SEMFs *in vitro*. The cells were incubated with IL-26 (100 ng/mL) for 12 h, and then IL-6 and IL-8 levels in supernatants were evaluated using ELISA. As shown Figure 4A, IL-26 induced a significant increase in the secretion of IL-6 and IL-8.

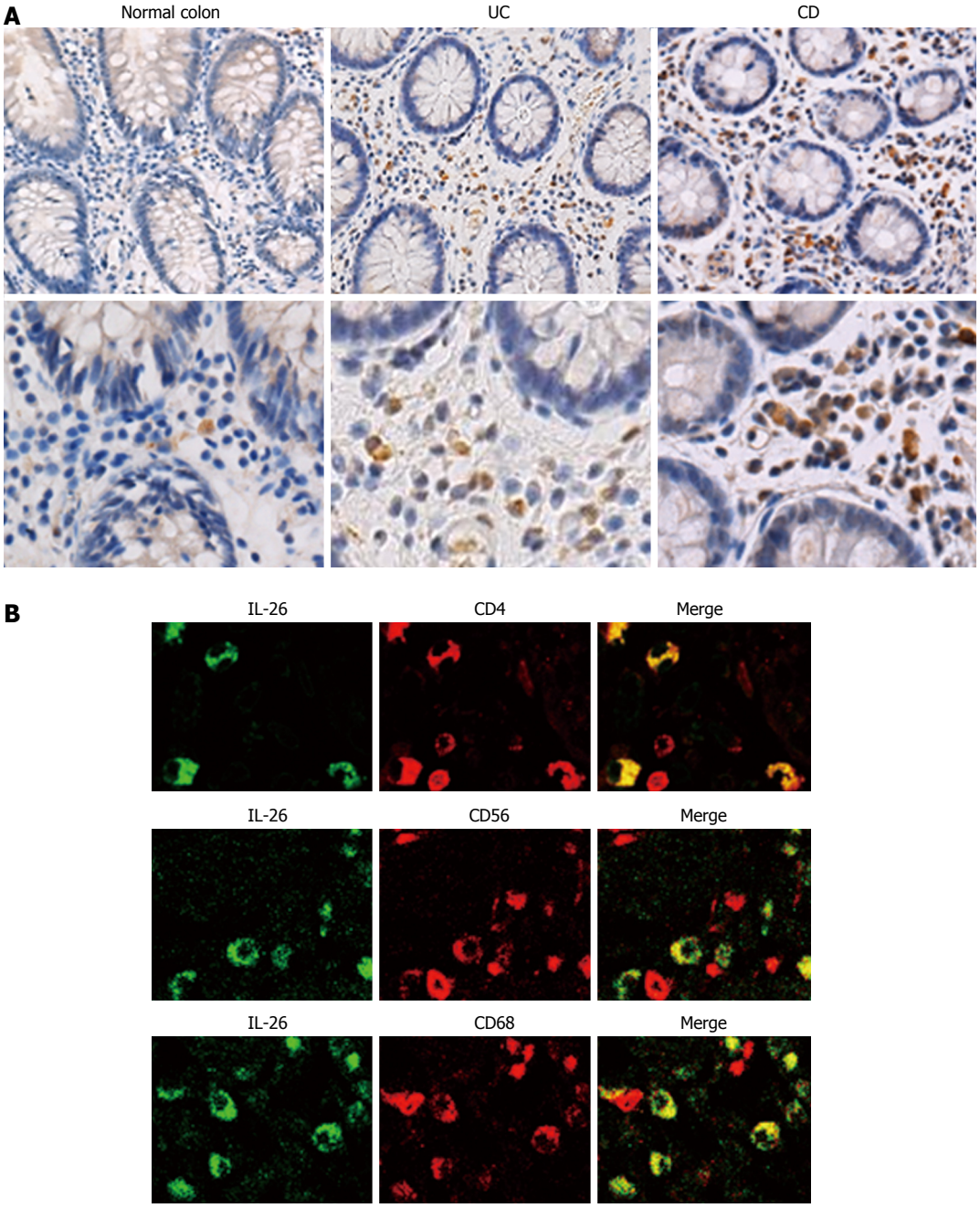

Figure 2 Immunohistochemical analyses of interleukin-26 in the inflamed mucosa of inflammatory bowel disease patients. A: Immunostaining for IL-26 in normal mucosa and inflamed mucosa of UC and CD. Pictures are shown from one of four independent samples with similar results. Magnification; upper panel × 200, lower panel × 400; B: Dual-colored immunofluorescence was used to determine the expression of IL-26 (green fluorescence) and CD4, CD56 and CD68 (red fluorescence) in inflamed mucosa of UC patients. Double-positive cells were detected by yellow fluorescence. Pictures are shown from one of four independent samples with similar results. IL: Interleukin; CD: Crohn's disease; UC: Ulcerative colitis.

These responses were also confirmed at the mRNA levels. IL-26 dose- and time-dependently induced the mRNA expression of IL-6 and IL-8 (Figure 4B and C).

Activation of STAT1 and STAT3 by IL-26 in human colonic SEMFs

It has been previously reported that the activation of STAT1 and STAT3 is induced by IL-26[16,22]. Therefore, we examined whether IL-26 induced the phosphorylation of STAT1 and STAT3 in human colonic SEMFs. IL-26 induced the phosphorylation of STAT1 and STAT3 as early as 5 min after stimulation with IL-26 (Figure 5A).

Involvement of STAT1 and STAT3 activation in IL-26-induced IL-6 and IL-8 was tested using siRNA specific for STAT1 and STAT3. As shown in Figure 5B, the siRNA specific for STAT1 and STAT3 significantly suppressed IL-26-induced mRNA expression of IL-6 and IL-8 effectiveness of siRNA for STAT1 and STAT3 is presented in Figure 6A. These findings indicated that

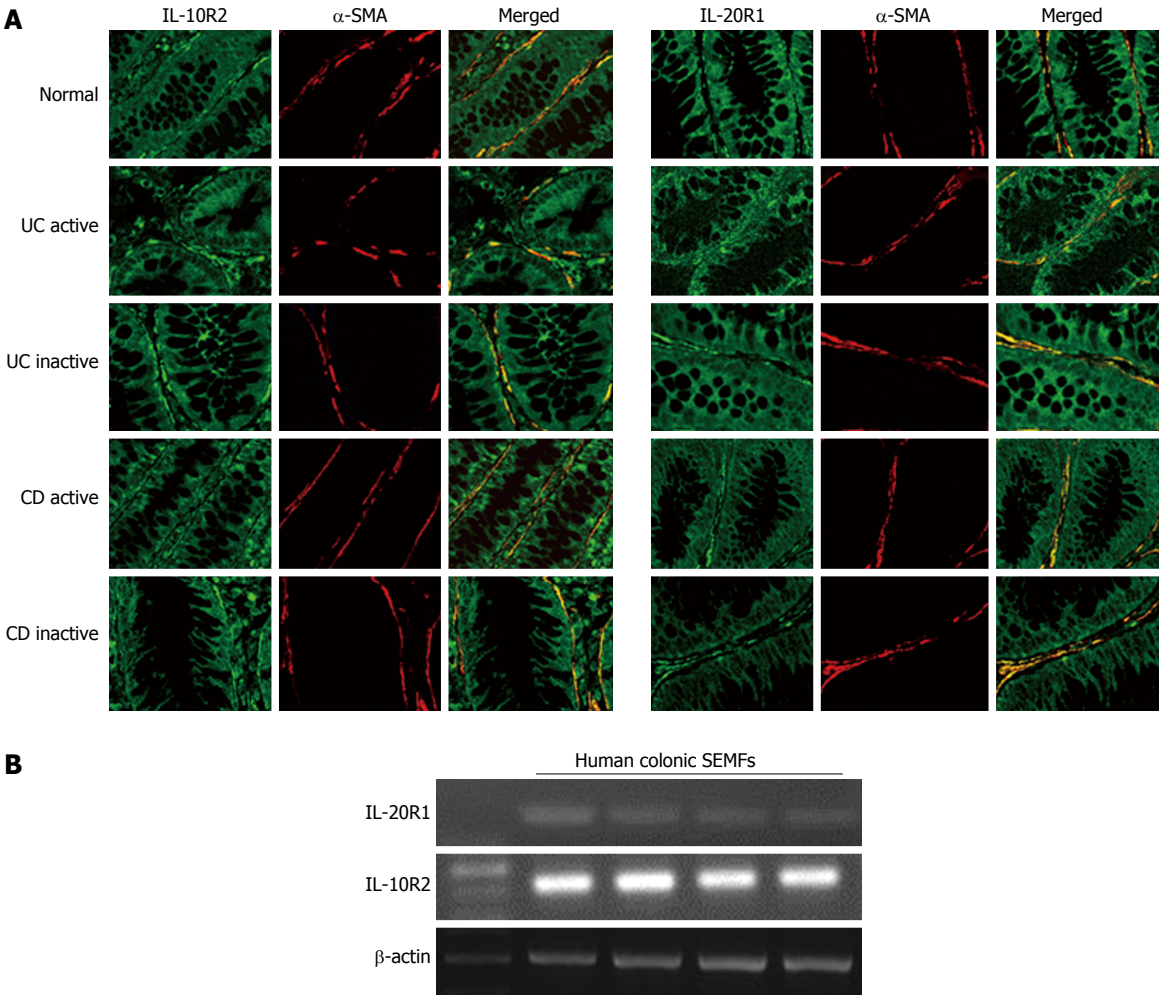

Figure 3 Immunohistochemical analyses of interleukin-26 receptor expression in normal and active/inactive inflammatory bowel disease mucosa. A: Dual-colored immunofluorescence was used to localize α-SMA (red fluorescence) and IL-26 receptor, IL-10R2 and IL-20R1 (green fluorescence). Dual positive immunostaining can be seen as yellow fluorescence in the merged images. Pictures are shown from one of four independent samples with similar results. Magnification × 200; B: The expression of IL-26 receptor in human colonic SEMFs isolated from four different surgical samples. The expression of IL-26 receptor was evaluated by RT-PCR. SMA: Smooth muscle actin; IL: Interleukin; SEMFs: Subepithelial myofibroblasts.

the activation of STAT1 and STAT3 is involved in the induction of IL-6 and IL-8 by the stimulation of IL-26 in human colonic SEMFs.

Activation of ERK1/2, SAPK/JNK1/2 and Akt by IL-26 in colonic SEMFs

The MAPKs and Akt are involved in the cytokine signaling in various kinds of cells. We examined whether IL-26 activates MAPKs and Akt using immunoblot analysis. As shown in Figure 5C, IL-26 induced a phosphorylation of MAPKs, including p42/44MAPK, SAPK/JNK, and p38MAPK, and Akt as early as 5 min after stimulation with IL-26. Moreover, MEK1/2 inhibitors (PD098059 and U0216), a p38 MAPK inhibitor (SB203580), a JNK inhibitor (JNK inhibitor 1) and a PI3K inhibitor (LY294002) significantly suppressed IL-26-induced IL-6 and IL-8 mRNA expression (Figure 5D). These findings indicate that the activation of MAPKs and the PI3K/Akt pathway is involved in the IL-26-induced IL-6 and IL-8 expression in human colonic SEMFs.

Activation of NF-κB and AP-1 by IL-26

The expression of a number of inflammatory genes is regulated by the activation of transcription factors such as NF-κB and AP-1[36]. In the nuclear proteins, the expression of NF-κB and phosphorylated c-Jun was clearly detected after IL-26 stimulation (Figure 7A). The siRNAs specific for NF-κBp65 and c-Jun (AP-1) significantly suppressed the mRNA expression of IL-6 and IL-8 effectiveness of siRNA for NF-κBp65 and c-Jun (AP-1) is presented in Figure 6B, (Figure 7B). These findings indicate an involvement of NF-κB and AP-1 activation in IL-26-induced IL-6 and IL-8 expression. As shown in Figure 7C, IL-26-induced NF-κBp65 phosphorylation was suppressed by PI3K inhibitor (LY294002), but was not affected by MAPKs. On the other hand, IL-26-induced AP-1 (c-Jun) phosphorylation was suppressed by both MAPKs and PI3K inhibitor. These findings indicate that PI3K, but not MAPKs, plays a role in IL-26-induced NF-κB activation, but that both MAPKs and PI3K activation are involved in IL-26-

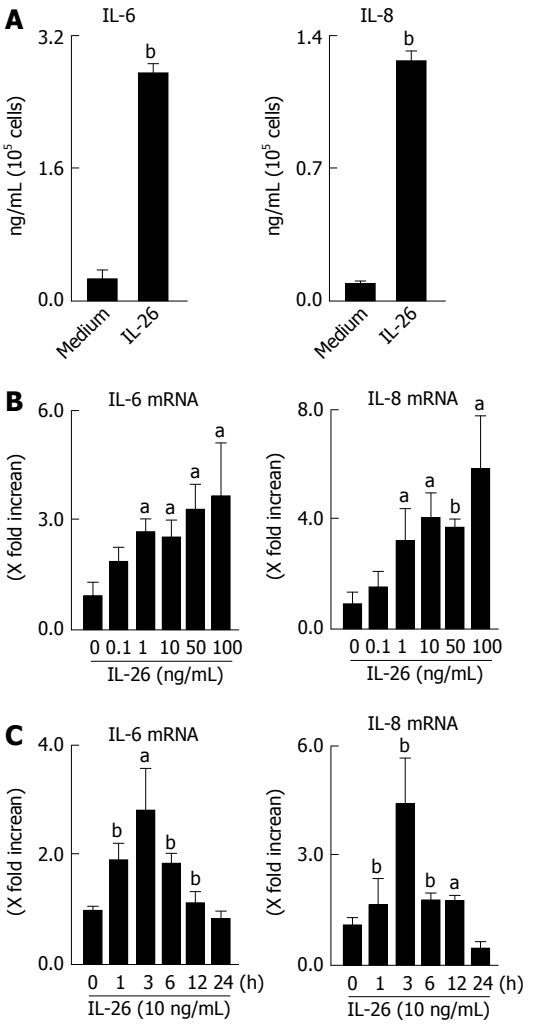

Figure 4 Secretion of interleukin-6 and interleukin-8 in response to interleukin-26 stimulation in human colonic subepithelial myofibroblasts. A: The cells were stimulated for 12 h with 100 ng/mL of IL-26, and the secretion of IL-6 and IL-8 in supernatants were determined using ELISA; B: Dose-dependent effects of IL-26 on IL-6 and IL-8 mRNA expression. Cells were stimulated for 3 h with increasing concentrations of IL-26, and the mRNA expression of IL-6 and IL-8 was then determined using real-time PCR. The mRNA expression of IL-6 and IL-8 was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone; C: The kinetics of IL-6 and IL-8 induction by IL-26. Human colonic SEMFs were stimulated with 100 ng/mL of IL-26 for the indicated predetermined times, and the mRNA expression of IL-6 and IL-8 was then determined using real-time PCR. The mRNA expression of IL-6 and IL-8 was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone (no stimulation). Data are expressed as mean ± SE of four independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. IL: Interleukin; SEMFs: Subepithelial myofibroblasts.

induced AP-1 activation.

DISCUSSION

In the present study, we demonstrated that: (1) the expression of IL-26 is enhanced in the inflamed mucosa of UC and CD patients; (2) human colonic SEMFs express IL-26 receptor complex IL-10R2/IL-20R1 *in vivo* and *in vitro*; (3) human colonic SEMFs secrete inflammatory mediators in response to IL-26 stimulation; and (4) IL-26 induced inflammatory mediators *via* the activation of STAT1/3 and MAPKs/PI3K followed by the activation of NF-κB/AP-1. These observations suggest that IL-26 plays a role in the pathophysiology of IBD.

In this study, we found that IL-26 mRNA expression was enhanced in the inflamed mucosa of UC and CD patients. Its expression was higher in the active mucosa of CD patients than in the active mucosa of UC patients. These findings are similar to our previous observation that mucosal mRNA expression of Th17 cytokines, such as IL-17 and IL-22, was enhanced in the inflamed mucosa of UC and CD patients[29,37]. The mRNA expression of either IL-17 or IL-22 was higher in CD patients than UC patients[29,37]. Th17 cells are now recognized as one of the cellular sources of IL-26[15]. These findings suggest that the IL-26-expressing $CD4^+$ T cells in this study are probably Th17 cells and that Th17 cells are more closely associated with the pathophysiology of CD patients than UC patients.

In the intestinal mucosa, $CD56^+NCR^+$ type 3 innate lymphoid cells (ILC3s), a subclass of $CD56^+$ $NKp44^+$ NK cells, have been reported to concomitantly express IL-22 and IL-26, especially following stimulation with IL-23[11]. This suggests that the IL-26-producing $CD56^+$ cells in our study may be NCR^+ ILC3s. Our observation of IL-26 expression by $CD68^+$ macrophages is supported by the recent report that $CD68^+$ macrophages are the main IL-26-producing cells in joints with rheumatoid arthritis[15]. Thus, our observations in this study indicate that various types of immune cells are producing IL-26 in the inflamed mucosa of IBD. Further investigation using more precise cellular markers should be performed to more clearly identify the cellular source of IL-26 in the inflamed mucosa of IBD.

There are some reports concerning the *in vivo* expression of IL-26 under normal and pathological conditions. Corvaisier *et al*[15] demonstrated a pathogenic role of IL-26 in rheumatoid arthritis on the basis of its capacity to induce pro-inflammatory cytokines. IL-23-induced IL-26 plays a role in the pathophysiology of psoriasis[8]. Moreover, Dambacher *et al*[16] have reported that IL-26 modulated proliferation and pro-inflammatory gene expression in colon cancer cells and that IL-26 expression was upregulated in active CD, suggesting a role of IL-26 in the innate host cell response during intestinal inflammation. In addition, a genome-wide association study identified IL-26 as one of the susceptibility genes associated with UC[38], suggesting a pathophysiological role of IL-26 in patients with UC. However, previous studies of the clinical role of IL-26 mainly focused on CD rather than UC[16]. In this study, we found that IL-26 was enhanced in the inflamed mucosa of patients with UC, as well as patients with CD. In addition, human colonic SEMFs were expressing functionally-active IL-10R2 and IL-20R1 and secreted inflammatory cytokines

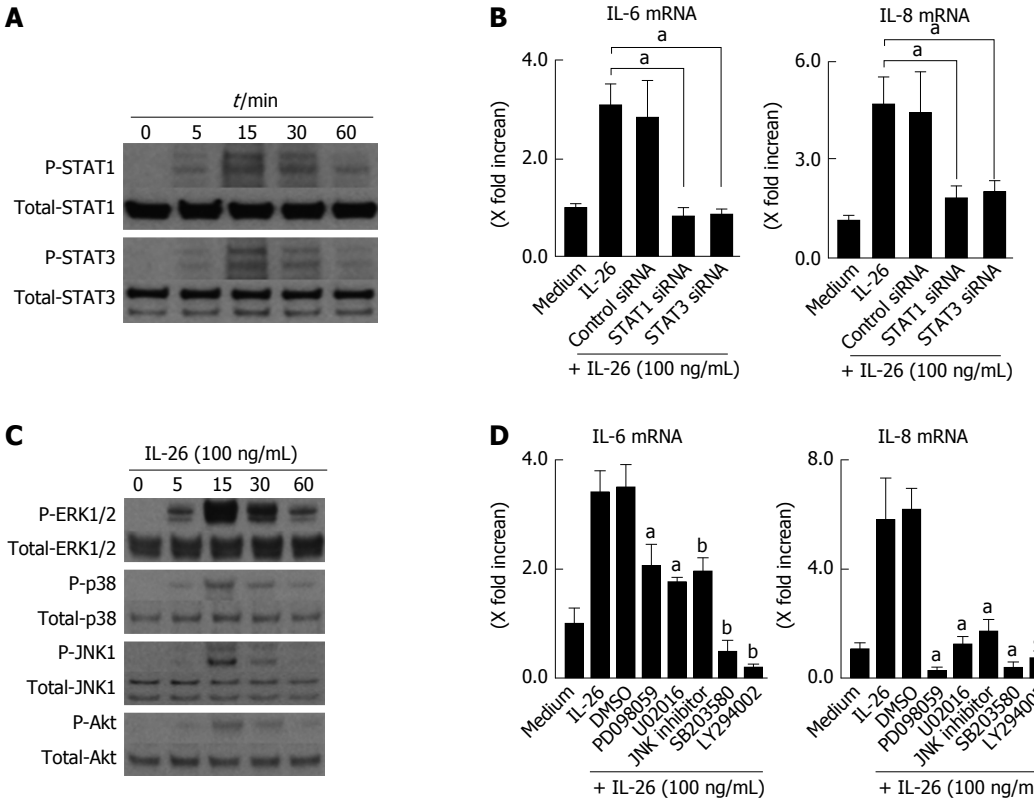

Figure 5 Involvement of STAT1, STAT3, MAPKs and PI3K/Akt activation in interleukin-26-stimulated interleukin-6 and interleukin-8 induction. A: STAT1 and STAT3 activation in response to IL-26. Human colonic SEMFs were stimulated with 100 ng/mL of IL-26 for the indicated pre-determined times, and the phosphorylation (P-) of STAT1 and STAT3 was evaluated by immunoblot analyses. The data are representative of two independent experiments; B: The effects of siRNAs for STAT1 and STAT3 on IL-26-induced mRNA expression of IL-6 and IL-8. Cells were transfected with siRNA for STAT1 and STAT3 or control siRNA, and were incubated for 3 h with or without 100 ng/mL of IL-26. The mRNA expression of IL-6 and IL-8 was then evaluated using real-time PCR. The mRNA expression of IL-6 and IL-8 was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone (no stimulation); C: MAPKs and PI3K/Akt activation in response to IL-26. Human colonic SEMFs were stimulated with 100 ng/mL of IL-26 for the indicated pre-determined times, and the phosphorylation (P-) of MAPKs and PI3K was evaluated by immunoblot analyses. The data are representative of two independent experiments; D: Effects of inhibitors of MAPKs and PI3K/Akt on IL-6 and IL-8 induction by IL-26 in human colonic SEMFs. The cells were pretreated with 10 μmol/L of a p38 MAPK inhibitor (SB203580) or an MEK1/2 inhibitor (U0216 or PD098059), or with 3 μmol/L of a JNK inhibitor (JNK inhibitor I) or 25 μmol/L of a PI3K inhibitor (LY294002) for 30 min, and were then incubated with or without 100 ng/mL of IL-26 for 3 h. The mRNA expression of IL-6 and IL-8 was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone. Data are expressed as mean ± SE of four independent experiments. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* IL-26 stimulation. IL: Interleukin; SEMFs: Subepithelial myofibroblasts.

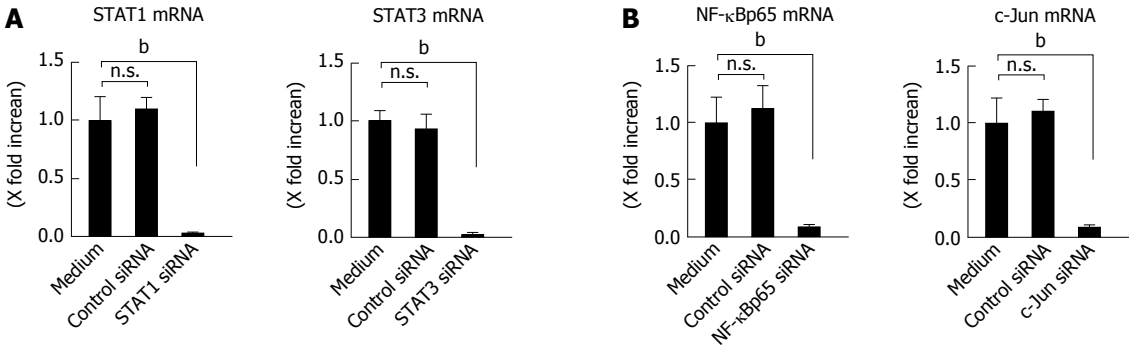

Figure 6 Effect of siRNA. A: Effect of siRNAs specific for STAT1 and STAT3 in human colonic subepithelial myofibroblasts. The cells were transfected with siRNAs specific for STAT1 and STAT3. A control siRNA was also used. The effect of siRNAs was evaluated using real-time PCR. The expression of STAT1 and STAT3 was significantly suppressed by specific siRNAs as compared to a control siRNA. STAT1 and STAT3 mRNA expression was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone (no stimulation); B: Effect of siRNAs specific for NF-κBp65 and c-Jun (AP-1) in human colonic subepithelial myofibroblasts. The cells were transfected with siRNAs specific for NF-κBp65 and c-Jun (AP-1). A control siRNA was also used. The effect of siRNAs was evaluated using real-time PCR. The expression of NF-κBp65 and c-Jun (AP-1) was significantly suppressed by specific siRNAs as compared to a control siRNA. The mRNA expression of NF-κBp65 and c-Jun (AP-1) mRNA was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone (no stimulation). Data are expressed as mean ± SE of four independent experiments. $^{b}P < 0.01$ *vs* medium. n.s.: Not significant.

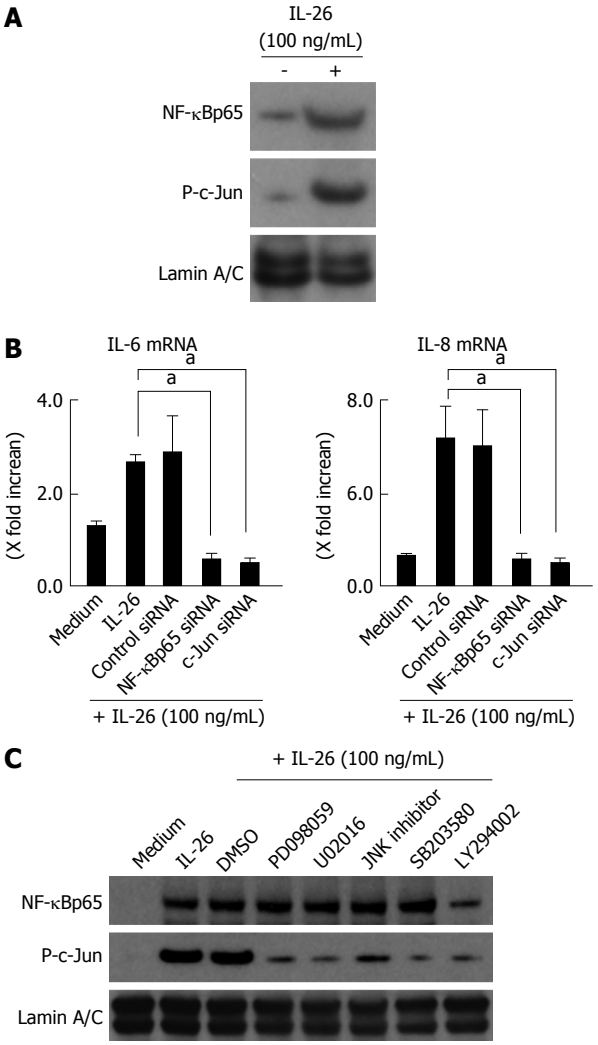

Figure 7 Involvement of nuclear factor-κB and AP-1 activation in the induction of inflammatory mediators induced by interleukin-26. A: Activation of NF-B and AP-1 (c-Jun) in response to IL-26 in human colonic SEMFs. The cells were stimulated with 100 ng/mL of IL-26 for 15 min, following which nuclear proteins were extracted and analyzed by immunoblotting for NF-κB (p65) and phosphorylated (P-) c-Jun. Lamin A/C was used as a loading control. The data are representative of two individual experiments; B: The effects of siRNAs specific for NF-κBp65 and c-Jun (AP-1) on IL-26-induced expression of IL-6 and IL-8 in human colonic SEMFs. The cells were transfected with siRNA specific for NF-κBp65, c-Jun (AP-1) or a control siRNA, and were incubated for 24 h with or without 100 ng/mL of IL-26. The mRNA expression of IL-6 and IL-8 was evaluated using real-time PCR. The mRNA expression of IL-6 and IL-8 mRNA was converted to a value relative to β-actin mRNA expression and presented as fold-increase relative to the results for medium alone (no stimulation). Data are expressed as mean ± SE of four independent experiments. $^{a}P < 0.05$ *vs* IL-26 stimulation; C: The effect of MAPKs and PI3K/Akt on the activation of NF-κBp65 and c-Jun (AP-1). The cells were pretreated with 10 μmol/L of a p38 MAPK inhibitor (SB203580) or an MEK1/2 inhibitor (U0216 or PD098059), or with 3 μmol/L of a JNK inhibitor (JNK inhibitor I) or 25 μmol/L of a PI3K inhibitor (LY294002) for 30 min, and were then incubated with or without 100 ng/mL of IL-26 for 15 min. Nuclear proteins were then extracted and analyzed by immunoblotting for NF-κBp65 and phosphorylated (P-) c-Jun. Lamin A/C was used as a loading control. The data are representative of two individual experiments. IL: Interleukin.

in response to IL-26. This phenomenon is supported by previous reports that the expression of IL-20R1 is restricted within non-hematopoietic cells[19,21]. These observations suggest an interaction between IL-26 and colonic SEMFs in inflammatory responses in the colonic mucosa. IL-26 may stimulate the induction of inflammatory mediators from colonic SEMFs and possibly contributes to the inflammatory responses in IBD mucosa. However, recent studies have revealed that Th17-derived cytokines, such as IL-17 and IL-22, possess conflicting (pro-inflammatory and protective) roles in the mucosa[39]. So, further investigations to determine whether IL-26 production is ultimately tissue protective or a significant source of tissue damage in IBD mucosa are required in the future.

There are a few reports concerning the signaling pathway of IL-26 in the colonic tissue. A previous report using transformed epithelial cell lines suggested that IL-26 induces activation of STAT1/3, ERK1/2, SAPK/JNK1/2 and Akt[16]. To our knowledge, this is the first report to state that IL-26 activates STAT1/3 and leads to the induction of IL-6 and IL-8 expression in non-transformed cells derived from human colon. We have also revealed that IL-26 induced the activation of MAPKs, including ERK1/2, p38MAPK and SAPK/JNKL1/2, and PI3K/Akt. Furthermore, we found that PI3K, but not MAPKs, plays a role in IL-26-induced NF-κB activation, but that both MAPKs and PI3K activation were required in IL-26-induced AP-1 activation. These results indicate that IL-26-induced inflammatory responses in the colonic mucosa are mediated by various signaling pathways including STAT1/3, MAPKs and PI3K/Akt, followed by the activation of NF-κB and AP-1.

In conclusion, we demonstrated that the expression of IL-26 is increased in the inflamed mucosa of IBD patients. In human SEMFs, IL-26 induced an activation of STAT1/3 and MAPKs/PI3K, leading to an activation of NF-κB and AP-1. Since the IL-26 gene has not been identified in rodents, the experiments using human colonic SEMFs will contribute to the investigation of the true role of IL-26 in gut inflammation.

COMMENTS

Background

Recent studies have reported that interleukin (IL)-26 is involved in the pathophysiology of chronic inflammatory disorders. Concerning inflammatory bowel disease (IBD), the pathological role of IL-26 has been reported in Crohn's disease (CD), but remains unclear in ulcerative colitis. Moreover, functional analysis of IL-26 has been studies using a transformed cell line, but there are no reports using primary culture cells.

Research frontiers

The authors found that the expression of IL-26 was enhanced in the inflamed mucosa of IBD as compared to in the normal mucosa. The cellular source of IL-26 in the inflamed mucosa are $CD4^{+}$ T cells, NK cells, and macrophages. Human colonic subepithelial myofibroblasts (SEMFs) are target cells of IL-26 in the mucosa of IBD.

Innovations and breakthroughs

This study revealed that IL-26 enhanced the induction of inflammatory mediators, IL-6 and IL-8, in the human colonic SEMFs. The inhibitors of IL-26

signaling pathway significantly suppressed the induction of IL-6 and IL-8. The inhibition of IL-26 signaling may lead to the suppression of intestinal inflammation.

Applications

The results of this study indicated that IL-26 may an important role in the pathogenesis of IBD. The authors suggested that IL-26 can a therapeutic candidate for IBD.

Terminology

IL-26 is a member of the IL-10 cytokine family. IL-26 is located on chromosome 12q5. IL-26 requires the heterodimeric receptors composed of IL-20R1 and IL-10R2.

Peer-review

The authors demonstrated that an increased expression of IL-26 in the inflamed mucosa of IBD patients and explored its possible pathway in IBD pathology. The study is well designed and the demonstration seems sufficient.

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P- Reviewer: Marzaban R, Pastorelli R, Reyes VE, Wu ZQ, Zimmer V
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5530-5537

DOI: 10.3748/wjg.v23.i30.5530

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

ORIGINAL ARTICLE

Basic Study

Autophagic cell death induced by reactive oxygen species is involved in hyperthermic sensitization to ionizing radiation in human hepatocellular carcinoma cells

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Author contributions: Yuan GJ designed the study and wrote the manuscript; Deng JJ performed the majority of experiments; Cao DD, Shi L, Chen X and Lei JJ performed some experiments of the study and data analysis; Xu XM provided partial financial support for this work and was involved in editing the manuscript.

Supported by Science and Technology Program of Chongqing, No. 2013-2-179.

Institutional review board statement: HepG2 cells were used in the study, and no specimens were taken clinically. Therefore, the ethic approval was not required.

Conflict-of-interest statement: No conflict of interest exists.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: March 15, 2017
Peer-review started: March 17, 2017
First decision: May 16, 2017
Revised: May 26, 2017
Accepted: July 4, 2017
Article in press: July 4, 2017
Published online: August 14, 2017

Abstract

AIM

To investigate whether autophagic cell death is involved in hyperthermic sensitization to ionizing radiation in human hepatocellular carcinoma cells, and to explore the underlying mechanism.

METHODS

Human hepatocellular carcinoma cells were treated with hyperthermia and ionizing radiation. MTT and clonogenic assays were performed to determine cell survival. Cell autophagy was detected using acridine orange staining and flow cytometric analysis, and the expression of autophagy-associated proteins, LC3 and p62, was determined by Western blot analysis. Intracellular reactive oxygen species (ROS) were quantified using the fluorescent probe DCFH-DA.

RESULTS

Treatment with hyperthermia and ionizing radiation significantly decreased cell viability and surviving fraction as compared with hyperthermia or ionizing radiation alone. Cell autophagy was significantly increased after ionizing radiation combined with hyperthermia treatment, as evidenced by increased formation of acidic vesicular organelles, increased expression of LC3II and decreased expression of p62. Intracellular ROS were also increased after combined

treatment with hyperthermia and ionizing radiation. Pretreatment with N-acetylcysteine, an ROS scavenger, markedly inhibited the cytotoxicity and cell autophagy induced by hyperthermia and ionizing radiation.

CONCLUSION

Autophagic cell death is involved in hyperthermic sensitization of cancer cells to ionizing radiation, and its induction may be due to the increased intracellular ROS.

Key words: Autophagic cell death; Hyperthermia; Ionizing radiation; Hepatocellular carcinoma; Reactive oxygen species

Core tip: Increased cell autophagy and intracellular reactive oxygen species (ROS), accompanied by decreased cell viability and surviving fraction, were observed in HepG2 cells treated with hyperthermia and ionizing radiation. Pretreatment with N-acetylcysteine, an ROS scavenger, markedly inhibited the above cytotoxicity and cell autophagy. The results suggest that autophagic cell death is involved in the hyperthermic sensitization to ionizing radiation, and its induction may be due to the increased intracellular ROS.

Yuan GJ, Deng JJ, Cao DD, Shi L, Chen X, Lei JJ, Xu XM. Autophagic cell death induced by reactive oxygen species is involved in hyperthermic sensitization to ionizing radiation in human hepatocellular carcinoma cells. *World J Gastroenterol* 2017; 23(30): 5530-5537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5530.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5530

INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cancer and the second leading cause of cancer-associated mortality in China[1]. A majority of patients with HCC present unresectable or advanced disease at the time of diagnosis; the choice of treatment is limited and the prognosis is poor[2]. With the development of radiotherapy techniques, including intensity-modulated radiotherapy (IMRT) and stereotactic body radiation therapy (SBRT), the use of radiotherapy has increasingly been adopted for the treatment of HCC[3]. Hyperthermia, elevation of temperature inside tumor up to 40-42 ℃, is an effective treatment modality for cancer[4]. Hyperthermia is a potent radiation sensitizer[5], and its combination with radiotherapy is a promising method for cancer treatment. Several studies have demonstrated the efficacy of combined treatment with radiotherapy and hyperthermia against HCC, head and neck cancer, breast cancer, and melanoma[6-9]. Hyperthermia can increase tumor perfusion and oxygenation, and inhibit repair of DNA damage in tumor cells, all of which may enhance tumor radiosensitivity[10,11]. However, the underlying mechanisms of the radio-sensitizing effect of hyperthermia are not fully elucidated.

Autophagy is an evolutionarily conserved process in which cellular organelles and long-lived proteins are sequestered into double-membrane vesicles, the autophagosomes, and subsequently delivered to the lysosomes to be degraded or recycled[12]. It can be induced by a variety of stimuli, such as nutrient deprivation, hypoxia, reactive oxygen species (ROS), protein aggregates, and damaged organelles[13]. Anticancer therapies, such as chemotherapy, radiotherapy and hyperthermia, are also shown to induce autophagy within tumor cells[14-16]. Studies have shown that increased basal autophagy is required for cells to survive after physical or chemical damage[17]. However, excessive autophagy can induce type II programmed cell death (autophagic cell death), a form of nonapoptotic cell death[18]. In the present study, we showed that autophagic cell death is involved in hyperthermic sensitization to ionizing radiation in human HCC cells, and its induction may be due to the increased intracellular ROS.

MATERIALS AND METHODS

Cell culture

Human HCC cell line HepG2 was obtained from Wuhan University Cell Center. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL streptomycin and 100 U/mL penicillin in a humidified 5% CO_2 atmosphere at 37 ℃.

Treatment of cells with hyperthermia and ionizing radiation

Water-bath warming was performed and the temperature was controlled at 43 ℃. Cell culture bottles were packaged and put into the water at 43 ℃ for 0.5 h. Then cells were irradiated with 4 Gy of 6 MV X-ray beam (field, 10 cm × 10 cm; source-to-surface distance, 100 cm; dose rate, 0.8 Gy/min), using a Varian Clinac 23iX (Varian Medical Systems, Inc.) at room temperature. The period between the two treatments was less than 1 h. After irradiation, the cells were immediately returned to the cell incubator and incubated at 37 ℃ for 72 h.

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] colorimetric assay was used to determine the survival rate of cells. After the above treatment, the cells were washed with phosphate buffered saline (PBS), and incubated with 1 × MTT at 37 ℃ for 4 h. Then the absorbance at 570 nm was measured, and the survival rate of the cells was calculated according to the following equation: survival rate = (experimental absorbance

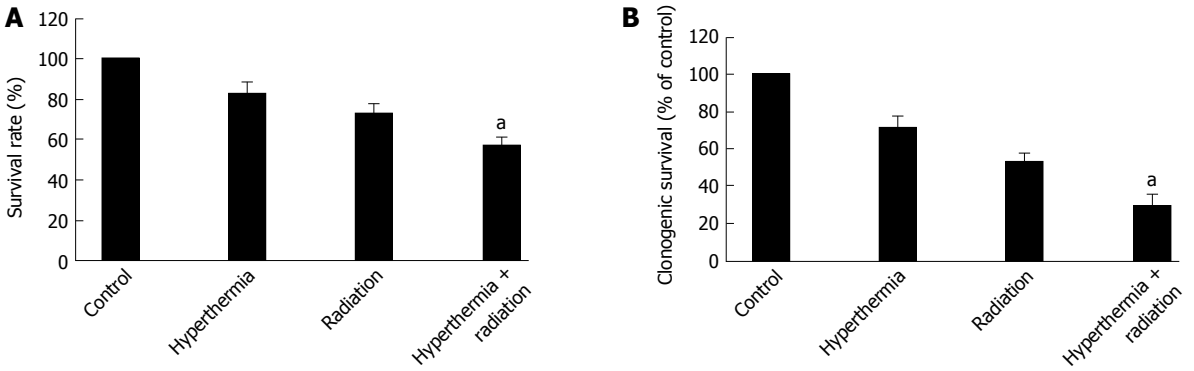

Figure 1 Hyperthermia enhances the cytotoxicity of ionizing radiation to hepatocellular carcinoma cells. HepG2 cells were treated with hyperthermia (43 ℃ for 0.5 h) followed by ionizing radiation (4 Gy). After 72 h of incubation, the cells were assessed for cell viability using MTT assay (A), or plated in dishes and incubated for clonogenic survival assay (B). The results are presented as the mean ± SD of three different experiments. $^{a}P < 0.05$ *vs* treatment of ionizing radiation alone.

value/control absorbance value) × 100%.

Clonogenic assay

After treatment with hyperthermia or ionizing radiation, the cells were trypsinized into single-cell suspension, plated in 60-mm dishes and incubated for 14 d to allow for colony growth. Then, the cells were fixed and stained with crystal violet, and colonies having at least 50 cells were counted using a microscope.

Acridine orange staining and flow cytometric analysis

Formation of acidic vesicular organelles, a morphological characteristic of autophagy, was detected using acridine orange staining. Cells were incubated with acridine orange (Invitrogen) at 1 μg/mL for 15 min. Red (650 nm, stained cytoplasmic vesicles) *vs* green (510-530 nm, stained nuclei) fluorescence (FL3/FL1) from cells illuminated with blue (488 nm) excitation light was measured with a FACScan flow cytometer (Beckman Coulter, Brea, CA, United States). The data are presented as the fold changes with an arbitrary setting of autophagy in cells without treatment of drug, hyperthermia or radiation.

Western blot analysis

Protein lysates were prepared using a total protein extraction kit (ProMab, SJ-200501), and stored at -20 ℃ until assay. The protein concentrations were assayed using the Bradford method. Equivalent aliquots of protein were separated by 10% SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS for 2 h at 37 ℃, washed with PBST (PBS with Tween 20) and incubated with rabbit polyclonal antibody against LC3 (dilution 1:500, CST) or p62 (dilution 1:500, CST) or mouse polyclonal antibody against GAPDH (glyceraldehyde 3-phosphate dehydrogenase, dilution 1:800, SANTA) at 4 ℃ overnight. After washing with PBST four times, the membranes were incubated with a secondary antibody (HRP-conjugated goat anti-rabbit IgG, SANTA, dilution 1:40000, for LC3 and p62; goat anti-mouse IgG, ZYMED, dilution 1:80000, for GAPDH) for 1 h at room temperature. The immunoreactive proteins were detected using an enhanced chemiluminescent detection system.

Determination of intracellular ROS

Intracellular ROS were measured using a ROS assay kit. After the above designated treatment, the cells were harvested and incubated with 10 μmol/L of DCFH-DA (a fluorescent probe, which may be oxidized by ROS in viable cells to 2′,7′-dichlorofluorescein, DCF) for 30 min at 37 ℃. After washing three times with PBS, DCF fluorescence was quantified with a multi-detection microplate reader (485 nm excitation and 535 nm emission).

Treatment of cells with N-acetylcysteine

N-acetylcysteine is an ROS scavenger. Cells were pretreated with N-acetylcysteine (10 mmol/L) for 1 h and then treated with hyperthermia or ionizing radiation as above.

Statistical analysis

Data were pooled from at least three independent experiments, and presented as mean ± SD unless otherwise indicated. Differences between groups were analyzed using one-way analysis of variance (ANOVA). All the statistical analyses were performed with SPSS13.0. *P* values less than 0.05 were considered statistically significant.

RESULTS

Hyperthermia enhances radiation cytotoxicity to HCC cells

The cytotoxicity induced by ionizing radiation with or without hyperthermia was assessed by MTT and clonogenic survival assays. As shown in Figure 1A, cell viability was decreased when the cells were treated with ionizing radiation or hyperthermia. The cell viability was significantly decreased after combined treatment with ionizing radiation and hyperthermia when

compared with each treatment alone. Furthermore, the clonogenic survival of the cells was also significantly decreased after ionizing radiation with hyperthermia as compared with radiation alone (Figure 1B).

Hyperthermia increases cell autophagy induced by ionizing radiation in HCC cells

Cell autophagy is characterized by the formation of numerous acidic vesicular organelles, which can be detected using acridine orange staining[19]. The acridine orange staining was quantified using flow cytometry. No obvious increase in cell autophagy was observed in HepG2 cells following 2 Gy ionizing radiation, or until 48 h after 4 Gy ionizing radiation. Therefore, in the present study, 4 Gy ionizing radiation was given to cells, and the cells were tested 72 h after ionizing radiation. As shown in Figure 2A and B, cell autophagy was significantly increased after combined treatment with ionizing radiation and hyperthermia compared with each treatment alone.

The expression of autophagy-related proteins was also detected by Western blot, among which the increase of LC3II protein and reduction of p62 protein are the hallmarks of the induction of autophagy[19]. A significant increase in LC3II expression and a reduction of p62 expression were observed in cells undergoing combined treatment as compared with those receiving ionizing radiation or hyperthermia treatment alone (Figure 2C).

N-acetylcysteine inhibits cytotoxicity and cell autophagy induced by hyperthermia and ionizing radiation in HCC cells

The intracellular ROS in ionizing radiation or hyperthermia-treated HepG2 cells were measured using a fluorescent probe, DCFH-DA. Treatment with ionizing radiation or hyperthermia induced an increase in intracellular ROS content, which was further increased by the combined treatment; however, the increase was completely inhibited by N-acetylcysteine, an ROS scavenger (Figure 3A).

To evaluate whether ionizing radiation or hyperthermia-induced autophagic cell death is related to intracellular ROS in HepG2 cells, pretreatment with N-acetylcysteine was performed. N-acetylcysteine pretreatment significantly improved cell viability in ionizing radiation or hyperthermia-treated HepG2 cells using MTT assay (Figure 3B). Furthermore, the autophagy rate was decreased (Figure 3C), and the expression of autophagy-related proteins, LC3II and p62, was reversed by N-acetylcysteine pretreatment in the ionizing radiation or hyperthermia-treated HepG2 cells (Figure 3D), suggesting that cell autophagy was inhibited.

DISCUSSION

In the past, radiotherapy was less considered in the treatment of HCC due to the belief that HCC is not a "radiosensitive" tumor and that radiotherapy is too "toxic" for the liver. However, recent studies have demonstrated that HCC is a radiosensitive tumor and its radiosensitivity is equivalent to poorly differentiated squamous cell carcinomas[20]. With the development of radiotherapy techniques, including IMRT and SBRT, a high radiation dose can be delivered to the tumor while the dose to the normal tissues can be simultaneously reduced. Therefore, at present radiotherapy is increasingly used for the treatment of HCC[3]. In the present study, we observed that the cell viability and clonogenic survival of HCC cells were decreased following ionizing radiation, suggesting that HCC cells are sensitive to radiation. Hyperthermia is a potent radiation sensitizer[5], and combined treatment with radiotherapy and hyperthermia has been shown to be efficacious against HCC[6]. Our study also showed that ionizing radiation combined with hyperthermia led to a significant decrease in the cell viability and clonogenic survival of HCC cells as compared with each treatment alone.

Autophagy is a major intracellular degradation mechanism for long-lived proteins and cytoplasmic organelles, the products of which are recycled to maintain cellular homeostasis. Increased basal autophagy is a cell survival mechanism in response to several stresses, such as nutrient deprivation, hypoxia, damaged mitochondria, protein aggregation and pathogens[17,21]. However, excessive autophagy may result in cell death, which is designated as type Ⅱ programmed cell death (autophagic cell death)[18]. Autophagy is frequently activated in tumor cells following anticancer therapies, such as chemotherapy, radiotherapy and hyperthermia[14-16]. Although several studies showed that induction of autophagy after irradiation acts as a protective and prosurvival mechanism and contributes to radioresistance in breast tumor cells and glioma cells[22,23], most experimental data have suggested that radiation-induced autophagy in cancer cell lines is related to cell death mechanisms and that autophagy-inducing agents may act as radiosensitizers[24]. In the present study, radiation-induced autophagy was significantly increased after combined treatment with hyperthermia in HCC cells, as evidenced by increased formation of acidic vesicular organelles, increased expression of LC3II and decreased expression of p62. The increased autophagy was accompanied with decreased cell viability and clonogenic survival in HCC cells, suggesting that autophagic cell death is involved in the enhancement of cellular radiosensitivity by hyperthermia.

ROS are small and highly reactive molecules that can oxidize proteins, DNA and lipids. They are generated as by-products of cellular metabolism primarily in mitochondria, and can also be produced in response to a variety of stimuli such as growth factors, inflammatory cytokines, ionizing radiation,

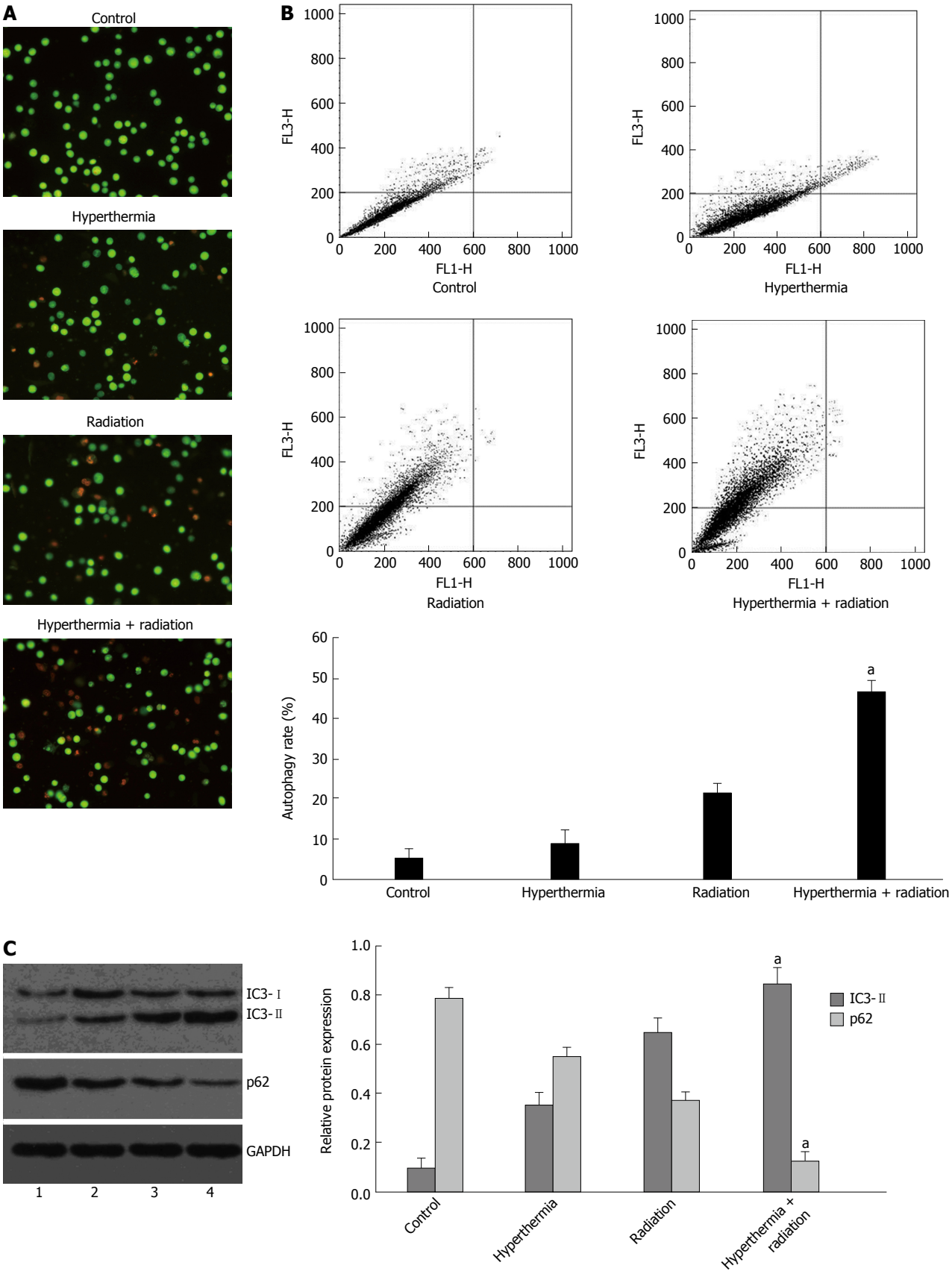

Figure 2 Hyperthermia increases ionizing radiation-induced autophagy in hepatocellular carcinoma cells. HepG2 cells were treated with hyperthermia (43 ℃ for 0.5 h) followed by ionizing radiation (4 Gy). After 72 h of incubation, the cells were assessed for autophagy by flow cytometry using acridine orange staining (A and B), or by Western blot analysis of LC3II and p62 expression (C) (Lane 1: Control; 2: Hyperthermia; 3: Radiation; 4: Hyperthermia + radiation). Results are presented as the mean ± SD of three different experiments, or representative of three different experiments. $^{a}P < 0.05$ *vs* treatment of ionizing radiation alone.

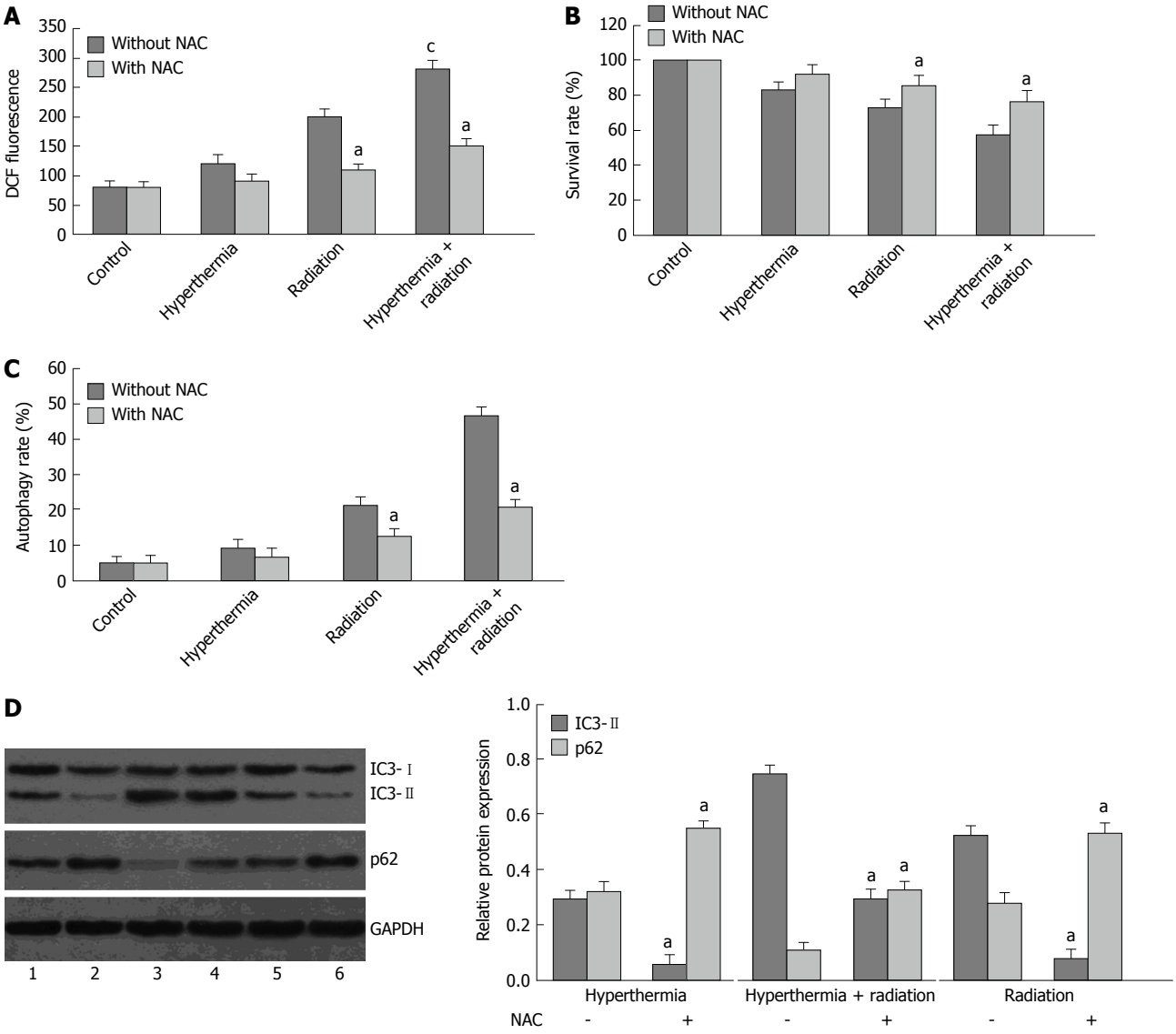

Figure 3 Intracellular reactive oxygen species formation and effect of N-acetylcysteine on the cytotoxicity and cell autophagy in hepatocellular carcinoma cells after treatment with ionizing radiation or hyperthermia. HepG2 cells were treated with hyperthermia (43 ℃ for 0.5 h) followed by ionizing radiation (4 Gy). After 72 h of incubation, the cells were assessed for intracellular ROS contents using DCFH-DA (A). HepG2 cells were pretreated with N-acetylcysteine (NAC, 10 mmol/L) for 1 h, and then treated with hyperthermia or ionizing radiation as above. After 72 h of incubation, the cells were assessed for cell viability using MTT assay (B), or for autophagy by flow cytometry using acridine orange staining (C) and by Western blot analysis of LC3II and p62 expression (D) (Lane 1: Hyperthermia; 2: Hyperthermia + NAC; 3: Hyperthermia + radiation; 4: Radiation; 5: Hyperthermia + radiation + NAC; 6: Radiation + NAC). Results are presented as the mean ± SD of three different experiments, or representative of three different experiments. $^{c}P < 0.05$ *vs* treatment of ionizing radiation or hyperthermia alone. $^{a}P < 0.05$ *vs* no pretreatment of NAC (*e.g.*, radiation *vs* NAC + radiation).

chemotherapy agents and toxins[25]. Hyperthermia has been also shown to induce production of ROS. A sharp increase in ROS generation has been observed after hyperthermia in a cellular model, using electron paramagnetic resonance spin trapping[26]. In the present study, we showed that intracellular ROS content was increased after treatment with ionizing radiation or hyperthermia, and was further increased by their combined treatment. Once ROS are produced, they act as signaling molecules that trigger diverse physiological and pathological responses, including induction of autophagy. Accumulating evidence has shown that oxidative stress is the converging point of many different inducers of autophagy, such as nutrient deprivation, viral infection and genotoxic stress[27]. In the present study, we found that pretreatment with N-acetylcysteine, an ROS scavenger, abolished the induction of autophagy by ionizing radiation or hyperthermia, and improved cell viability of HepG2 cells after the above treatment. These facts suggest that the induction of autophagic cell death by ionizing radiation and hyperthermia treatment in HCC cells is due to the increased intracellular ROS.

In conclusion, autophagic cell death is involved in hyperthermic sensitization of cancer cells to ionizing radiation, and its induction may be due to the increased

intracellular ROS.

COMMENTS

Background

With the development of radiotherapy techniques, radiotherapy has increasingly been used for the treatment of hepatocellular carcinoma (HCC). Hyperthermia is a useful adjuvant to radiation therapy in the treatment of many cancers, and it is a potent radiation sensitizer. However, the underlying mechanisms of the radio-sensitizing effect of hyperthermia are not fully elucidated. Autophagy is a major intracellular degradation mechanism for long-lived proteins and cytoplasmic organelles, and excessive autophagy may result in cell death, which is designated as type Ⅱ programmed cell death (autophagic cell death). The role autophagic cell death in hyperthermic sensitization to ionizing radiation has not been explored.

Research frontiers

Many studies have shown that radiation-induced autophagy is related to cell death mechanisms in cancer cell lines and that autophagy-inducing agents may act as radiosensitizers. Oxidative stress has been proven to be the converging point of many different inducers of autophagy, such as nutrient deprivation, viral infection and genotoxic stress.

Innovations and breakthroughs

The novel findings of this study are that autophagic cell death is involved in hyperthermic sensitization to ionizing radiation, and its induction may be due to the increased intracellular reactive oxygen species.

Applications

The study provides a novel theoretical basis for hyperthermia in the treatment of malignant tumors, especially combined with radiotherapy.

Terminology

Autophagy is an evolutionarily conserved process in which cellular organelles and long-lived proteins are sequestered into double-membrane vesicles, the autophagosomes, and subsequently delivered to the lysosomes to be degraded or recycled. Autophagic cell death is a kind of nonapoptotic programmed cell death (also known as type Ⅱ programmed cell death), characterized by using autophagosome to degrade cell content in dying cells.

Peer-review

HCC is still the object of research and this paper provides some information on the mechanism of hyperthermic sensitization of cancer cells to ionizing radiation.

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P- Reviewer: Desiderio J, Sun BC **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Zhang FF

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5538-5548

DOI: 10.3748/wjg.v23.i30.5538

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

ORIGINAL ARTICLE

Basic Study

Yangzheng Sanjie decoction regulates proliferation and apoptosis of gastric cancer cells by enhancing let-7a expression

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Author contributions: Deng HX performed the majority of experiments and drafted the manuscript; Yu YY conducted the molecular assays and assisted in writing the manuscript; Zhou AQ, Zhu JL and Chen WQ participated in the animal experiments and prepared the drug-containing serum; Luo LN performed the high-performance liquid chromatography analysis; Chen WQ and Hu L collected medical records and tissue specimens; Deng HX and Chen GX designed and coordinated the research, analyzed the data and revised the manuscript.

Supported by the National Natural Science Foundation of China, No. 81273735 and No. 81373563; Science and Technology Planning Project of Guangdong Province, China, No. 2016A020215135 and No. 2013B021800169; and The Specific Research Fund for TCM Science and Technology of Guangdong Provincial Hospital of Chinese Medicine, China, No. YN2014ZH05.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Guangzhou University of Chinese Medicine (No. 2013050). All operations were performed according to international guidelines concerning the care and treatment of experimental animals.

Institutional review board statement: This study was reviewed and approved by the Institutional Review Board of the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

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

Conflict-of-interest statement: The authors declare that there are no conflicts of interest related to this study.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: April 22, 2017
Peer-review started: April 25, 2017
First decision: June 1, 2017
Revised: June 20, 2017
Accepted: July 4, 2017
Article in press: July 4, 2017
Published online: August 14, 2017

Abstract

AIM

To explore the let-7a-mediated anti-cancer effect of Yangzheng Sanjie decoction (YZSJD) in gastric cancer (GC) cells.

METHODS

YZSJD-containing serum (YCS) was prepared using traditional Chinese medicine serum pharmacology methods. After YCS treatment, cell proliferation and apoptosis were assessed by cell counting kit-8 assay and flow cytometry, respectively, and miRNA expression profiles were determined using qPCR arrays. Let-7a expression was examined by *in situ* hybridization in GC tissues and by qPCR in GC cells. c-Myc protein expression was detected by immunohistochemistry in GC tissues, and by Western blot in cell lines.

RESULTS

YZSJD significantly inhibited proliferation and induced apoptosis in AGS and HS-746T GC cells. After treatment with YCS, the miRNA expression profiles were altered and the reduced let-7a levels in both cell lines were up-regulated, accompanied by a decrease in c-Myc expression. Moreover, decreased let-7a expression and increased c-Myc expression were observed during the progression of gastric mucosa cancerization.

CONCLUSION

YZSJD inhibits proliferation and induces apoptosis of GC cells by restoring the aberrant expression of let-7a and c-Myc.

Key words: Gastric cancer; Let-7a; c-Myc; Proliferation; Apoptosis; Yangzheng Sanjie decoction

Core tip: Let-7a reduction plays an important role in gastric tumourigenesis through the derepression of c-Myc expression. Our data demonstrate that Yangzheng Sanjie decoction (YZSJD) inhibits proliferation and induces apoptosis in gastric cancer (GC) cells by regulating the aberrant expression of let-7a and c-Myc. These findings provide new evidence that YZSJD has therapeutic potential in GC treatment and that miRNA regulation may be a novel molecular mechanism through which Chinese herbal medicine exhibits anti-cancer activity.

Deng HX, Yu YY, Zhou AQ, Zhu JL, Luo LN, Chen WQ, Hu L, Chen GX. Yangzheng Sanjie decoction regulates proliferation and apoptosis of gastric cancer cells by enhancing let-7a expression. *World J Gastroenterol* 2017; 23(30): 5538-5548 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5538.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5538

INTRODUCTION

Gastric cancer (GC) is one of the leading causes of cancer-related deaths in the world[1]. The low early diagnosis rate of GC and the limited treatment options for advanced GC are the major reasons for the high mortality rate in GC patients. Considering the narrow therapeutic window, long-term drug resistance and toxic side effects of chemotherapeutic agents, safer and more effective therapies should be developed to improve the prognosis of GC.

As an important source of novel agents with pharmaceutical potential, Chinese herbal medicines have become increasingly popular in cancer treatment as alternative and complementary therapy modalities[2-6]. One Chinese medicine formula, Yangzheng Sanjie decoction (YZSJD), which contains the ingredients *Astragali Radix, Scutellariae Barbatae Herba, Arisaematis Rhizoma Preparatum, Citri Sarcodactylis Fructus, Cremastrae Pseudobulbus* and *Curcumae Longae Rhizoma,* has shown good clinical effects in the treatment of chronic atrophic gastritis with precancerous lesions[7]. Several components of YZSJD have recently been reported to exert antiproliferative effects in several cancer cell lines[8-10]. However, the potential role of YZSJD in the treatment of GC and the precise mechanisms that may be involved in the proliferation and apoptosis of GC cells have not yet been clearly addressed.

Increasing evidence has revealed that microRNAs (miRNAs) play critical roles in the initiation, progression and aggressiveness of human cancers and may be potential therapeutic targets for malignancies. MiRNAs are a class of small noncoding RNAs of 21-23 nucleotides that negatively regulate gene expression by base-pairing with the 3′-untranslated regions of their target messenger RNAs[11,12]. The miRNA let-7 is down-regulated in many cancer types compared with normal tissue, indicating that the let-7 family serves as tumour suppressors[13,14]. Involved in the complex regulation of c-Myc, let-7a participates in the genesis and maintenance of c-Myc-dysregulated cancers[15]. Kim *et al*[16] demonstrated that let-7 inhibits c-Myc expression by targeting the c-Myc 3′-UTR in a HuR-let-7 interdependent manner. Let-7b increased drug sensitivity in chemotherapy-resistant GC cells by targeting c-Myc[17]. A more recent study revealed that let-7a down-regulates the expression of PKM2 by regulating the expression of c-Myc and hnRNPA1 and therefore inhibits the proliferation, migration and invasion of GC cells[18].

In a previous clinical study, we found that YZSJD can effectively slow, block or reverse the progression of GC precancerous lesions[19]. In this study, we aimed to explore the miRNA-mediated anticancer effect of YZSJD *in vitro*. The present study was therefore designed to evaluate cell proliferation and apoptosis in

GC cells treated with YZSJD-containing serum (YCS) and to screen and verify the potential miRNA targets. Furthermore, we detected the expression of let-7a and its target gene c-Myc in GC tissues, matched gastric precancerous tissues and normal gastric mucosa tissues to investigate the role of let-7a in the progression of gastric mucosa cancerization.

MATERIALS AND METHODS

Preparation and quality control of YZSJD

YZSJD, a Chinese herbal compound prescribed by Dr. Geng-Xin Chen at the Second Affiliated Hospital of Guangzhou University of Chinese Medicine, is composed of *Astragali Radix* 15 g, *Codonopsis Radix* 15 g, *Scutellariae Barbatae Herba* 15 g, *Arisaematis Rhizoma Preparatum* 10 g, *Citri Sarcodactylis Fructus* 10 g, *Cremastrae Pseudobulbus* 10 g, *Curcumae Longae Rhizoma* 10 g and *Curcumae Rhizoma* 15 g. All the medicinal materials used to prepare formulae were purchased from Kangmei Pharmaceutical Co., Ltd. (Guangzhou, China) and were identified by two pharmacognosy experts. The herbs (100 g) were soaked in distilled water (1000 mL) and boiled for 30 min twice, and then the extracts were filtered, mixed and centrifuged. The upper layer was concentrated to 0.9 g crude extract per millilitre in a rotary evaporator (SENCO, China) and stored at -20 ℃ for future use. To establish quality control standards for YZSJD, the crude extract preparation and subsequent high-performance liquid chromatography (HPLC) determination were repeated ten times. The similarity of the HPLC fingerprints of 10 batches of YZSJD samples was assessed using the Computer-Aided Similarity Evaluation System for Chromatographic Fingerprint of TCM (Chinese Pharmacopoeia Commission, version 2004A).

Preparation of YCS

Forty male SD rats (SPF grade, weighing 250 ± 20 g) were purchased from the laboratory animal centre of Southern Medical University. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. Subsequently, the rats were randomly and equally divided into a YZSJD group and a Control group. Animals in the YZSJD group were gavaged with an equivalent dose of YZSJD (9 g/kg), while those in the Control group were administered the same volume of normal saline once daily. On the seventh day, blood was drawn from the abdominal aorta 1 h after feeding, and the serum was isolated. Each group of sera was mixed, sterilized by filtration and inactivated at 56 ℃ before being stored at -20 ℃. These experiments were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Guangzhou University of Chinese Medicine, and efforts were made to minimize animal suffering.

Cell culture

The human GC cell lines AGS and HS-746T were purchased from the American Type Culture Collection (Manassas, VA, United States), the cell lines MKN-45 and SGC-7901 were obtained from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China), and the human immortalized gastric mucosa cell line GES-1 was provided by the Beijing Institute for Cancer Research. The cells were cultured in RPMI-1640 medium (HyClone, United States) supplemented with 10% foetal bovine serum (HyClone, United States) and maintained in a humidified incubator with 5% CO_2 at 37 ℃.

Cell groups and cell treatments

AGS and HS-746T cells were suspended and seeded in 96-well plates at a density of 6000 cells/well or in 6-well plates at a density of 20000 cells/well. The cells were divided into a YZSJD group and a Control group. After 12 h of culture, the cells in the YZSJD group were treated with 10% YCS, while those in the Control group were treated with 10% normal rat serum.

Cell proliferation assay

The effects of YZSJD on AGS and HS-746T cell proliferation were estimated using the Cell Counting Kit-8 (CCK-8) assay (Jingxin, China). After 24, 48 or 72 h of incubation with YCS or normal rat serum, 10 μL of CCK-8 solution was added to each well of a 96-well plate, followed by a 2-h incubation in the dark. Cell proliferation was evaluated by the absorbance of each well at 450 nm, which was measured with a VICTOR X5 Multilabel Plate Reader (PerkinElmer, United States).

Cell apoptosis assay

The effects of YZSJD on apoptosis were determined by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, United States). After 48 h of incubation with YCS or normal rat serum, cells in 6-well plates were harvested and resuspended in 1 × binding buffer at a concentration of 1 × 10^6 cells/mL. Then, 5 μL of Annexin V-FITC and 10 μL of propidium iodide were added to 100 μL of the cell suspension. The cells were incubated for 15 min in the dark before 400 μL of 1 × binding buffer was added. The samples were analysed by flow cytometry within 1 h.

MiRNA PCR array

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Contaminating DNA in the RNA preparations was removed with DNase I, and the RNA was purified using an RNeasy MinElute Cleanup Kit (Qiagen, Germany). The RNA quantity and purity were assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States), and RNA integrity was examined by denaturing

Table 1 Clinical and pathological characteristics of gastric cancer patients

Characteristic	*n* (%)
Age (yr)	
< 60	6 (54.5)
⩾ 60	5 (45.5)
Sex	
Male	8 (72.7)
Female	3 (27.3)
Lymph node metastasis	
Negative	6 (54.5)
Positive	5 (45.5)
Histopathology	
Adenocarcinoma	11 (100)
Others	0 (0)
Grade	
Well/moderately differentiated	5 (45.5)
Poorly differentiated	6 (54.5)
Stage	
Early	2 (18.2)
Advanced	9 (81.8)
Surgical therapy	
Negative	0 (0)
Positive	11 (100)

agarose gel electrophoresis.

The expression of mature miRNAs was detected using ExiLENT SYBR Green master mix (Exiqon, Denmark) and the microRNA Ready-to-Use PCR, Human panel Ⅰ + Ⅱ (V4.M) (Exiqon, Denmark), according to the manufacturer's instructions. Briefly, the template RNA was reverse transcribed using a Universal cDNA Synthesis Kit (Exiqon, Denmark), and the reverse transcription products were then amplified and detected on a 7900 Real-Time PCR System (Applied Biosystems, United States) using the following thermocycler conditions: denaturation at 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 10 s and 60 ℃ for 1 min.

Data analysis was performed with the GenEx qPCR analysis software (www.exiqon.com/mirna-pcranalysis). U6 snRNA was used as an endogenous control. The fold change for each miRNA was calculated as $2^{-\Delta\Delta Ct}$. We filtered out raw data for which the cycle threshold values were greater than 30 from the 372 total human miRNAs, and the differentially expressed miRNAs with a fold-change ⩾ 1.5 in at least one cell line were included in the further analyses.

qRT-PCR

Total RNA was isolated from AGS and HS-746T cells with Trizol reagent (Invitrogen, CA, United States). The RNA concentration and purity were assessed using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific, United States). Mature let-7a was reverse transcribed to cDNA using a PrimeScript RT reagent kit (TaKaRa, Japan), and the specific cDNA was amplified using a SYBR Premix Ex Taq II kit (TaKaRa, Japan) according to the manufacturer's instructions. The Bulge-Loop hsa-let-7a-5p qRT-PCR Primer Set for reverse transcription and quantitative PCR was purchased from Ribobio Biotechnology (Guangzhou, China). The reverse transcription conditions consisted of an initial incubation at 42 ℃ for 15 min followed by 85 ℃ for 5 s in a T100 PCR instrument (Bio-Rad, United States). The qPCR amplification was performed as follows: 95 ℃ for 30 s, followed by 40 cycles of 95 ℃ for 5 s and 60 ℃ for 30 s on a 7500 Real-Time PCR System (Applied Biosystems, United States). U6 was used as an endogenous control, and the $\Delta\Delta Ct$ method was used for let-7a quantification.

Western blot

Cells were lysed in RIPA buffer (Beyotime, China) to extract total cellular proteins. Equal amounts of protein for each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, United States). After being blocked with WB blocking solution (Beyotime, China) for 1 h, the membranes were incubated with a rabbit c-Myc monoclonal antibody (Cell Signaling Technology, United States) or a mouse β-actin monoclonal antibody (Boster, China) at 4 ℃ overnight. The next day, the membranes were incubated with either a goat anti-rabbit IgG or a goat anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (Boster, China) at 37 ℃ for 1 h. Immunoreactivity was visualized using an enhanced chemiluminescence reagent (Beyotime, China).

Tissue specimens

Tissue from 11 patients with pathologically diagnosed GC who underwent radical resection without radiotherapy and chemotherapy at the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, Guangdong province, China) from May 2014 to November 2014 was collected. The diagnosis and histopathologic type of GC were determined according to the rules of the Japanese Gastric Cancer Association[20]. The clinical and pathological characteristics of the patients are shown in Table 1. All study participants provided written informed consent and donated a piece of GC tissue, matched tissue adjacent to the carcinoma and distal normal gastric tissue. The samples were fixed in 4% paraformaldehyde immediately after surgery and then embedded in paraffin. Histopathology was confirmed independently by two experienced pathologists unaware of the patients' clinical history. The present study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine.

In situ hybridization

In situ hybridization was performed to detect let-7a in paraffin-embedded tissue sections using the Enhanced Sensitive ISH Detection Kit (Boster, China)

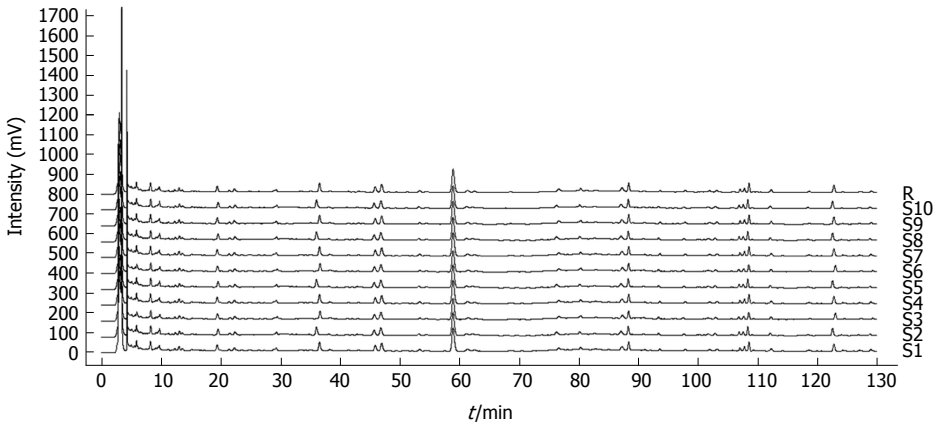

Figure 1 Yangzheng Sanjie decoction high-performance liquid chromatography fingerprints. High-performance liquid chromatography (HPLC) analysis was performed to determine the chromatographic fingerprints of Yangzheng Sanjie decoction. Analysed with a Computer-Aided Similarity Evaluation System, the similarity scores of the HPLC fingerprints of 10 batches of samples were above 0.95.

and DIG-labelled hsa-let-7a miRCURY LNA Detection probe (Exiqon, Denmark). After pretreatment and enzymatic digestion, slides were incubated with let-7a hybridization solution overnight at 4 ℃. After a 30 min blocking step, the slides were treated with an anti-digoxigenin antibody for 1 h. Then, SABC-POD solution was added to the slides for 20 min at 37 ℃. Next, the slides were treated with biotin peroxidase for 20 min and diaminobenzidine (DAB) chromogenic reagent (Boster, China) for 20 min. Counterstaining was performed with haematoxylin. Images were taken on an Olympus BX53 microscope equipped with a digital camera.

Signals were semi-quantitatively evaluated based on the cytoplasmic staining intensity and the percentage of positive cells. Staining intensity was divided into four levels: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong); the percentage of positive cells was graded as 0 (none), 1 (< 10%), 2 (10%-50%), 3 (50%-80%) and 4 (> 80%). A total *in situ* hybridization (ISH) score was calculated by multiplying the scores of intensity and percentage. According to the scores, signals were assessed as follows: - (≤ 1), + (2 to 4), ++ (4 to 8) and +++ (≥ 9).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on paraffin sections using a ready-to-use rabbit anti-human c-Myc monoclonal antibody (MXB, China). The sections were first deparaffinized in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase was blocked with 3% H_2O_2 for 10 min, and antigen retrieval was carried out with sodium citrate buffer at 0.1 MPa and 121 ℃ for 5 min. After a blocking step using 5% BSA, slides were separately incubated with 50 μL of primary antibody at 4 ℃ overnight. Then, they were visualized by incubation with a biotin-conjugated secondary antibody followed by streptavidin and DAB (Boster, China). Counterstaining was performed with haematoxylin. The c-Myc score of each sample was based on the nuclear staining. The data were evaluated as described above.

Statistical analysis

All reactions were performed in triplicate. Measurement data are expressed as the mean ± SD, and statistical analyses were performed using one-way analysis of variance. Ordinal data were analysed by Radit analysis. $P < 0.05$ was considered statistically significant.

RESULTS

YZSJD HPLC fingerprints

To establish quality control standards for YZSJD, HPLC was used to analyse 10 batches of YZSJD samples. As illustrated in Figure 1, the YZSJD HPLC fingerprints consisted of 22 characteristic peaks. The similarity scores of the fingerprinting profiles of the 10 batches of samples were above 0.95, which indicated the consistency and stability of the extracts and the preparation procedure.

YZSJD inhibits GC cell proliferation

To investigate the biological effect of YZSJD on GC progression *in vitro*, AGS and HS-746T GC cells were treated with YCS. We evaluated cell proliferation using the CCK-8 assay. As shown in Figure 2, YCS significantly decreased the viability of AGS and HS-746T cells in a time-dependent manner.

YZSJD induces apoptosis of GC cells

Next, we assessed cell apoptosis using flow cytometry. The early apoptosis rates of the two cell lines were significantly increased after treatment with YCS ($P < 0.01$). After 48 h treatment with YCS, the early apoptosis rates reached 9.97% ± 2.35% (AGS) and 10.9% ± 0.85% (HS-746T), while in the Control groups, the proportions of Annexin V^+/PI^- cells were only 1.67% ± 0.23% (AGS) and 1.8% ± 0.27% (HS-746T) (Figure 3).

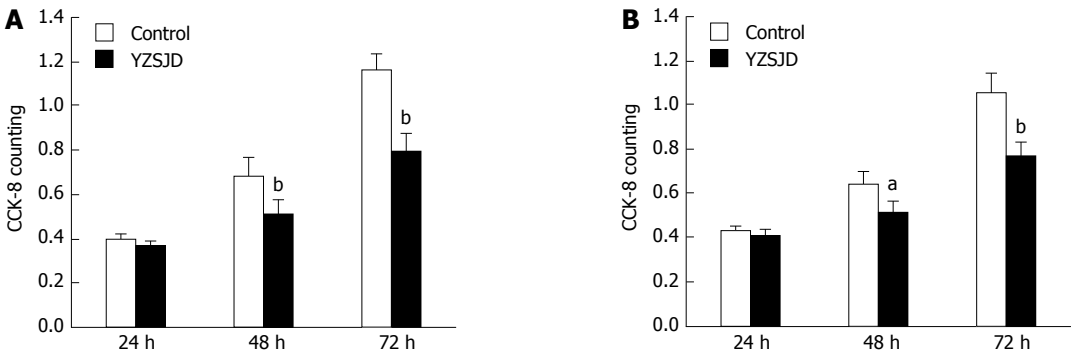

Figure 2 Yangzheng Sanjie decoction inhibits gastric cancer cell proliferation in a time-dependent manner. The viability of AGS (A) and HS-746T cells (B) after treatment with YCS was measured using a CCK-8 assay. [a]*P* < 0.05 *vs* the control group; [b]*P* < 0.01 *vs* the control group. YCS: YZSJD-containing serum; YZSJD: Yangzheng Sanjie decoction; CCK-8: Cell Counting Kit-8.

Table 2 Differentially expressed microRNAs in gastric cancer cells treated with Yangzheng Sanjie decoction

miRNA ID	Fold change (YZSJD/control)	
	AGS	HS-746T
hsa-let-7a-5p	1.54	1.80
hsa-let-7d-3p	1.66	1.53
hsa-let-7f-5p	1.68	2.70
hsa-miR-7-5p	1.56	2.03
hsa-miR-15a-5p	1.66	2.04
hsa-miR-15b-5p	1.62	3.26
hsa-miR-21-3p	1.86	1.75
hsa-miR-22-3p	1.58	1.78
hsa-miR-22-5p	1.74	1.60
hsa-miR-25-3p	1.74	1.76
hsa-miR-26b-5p	1.64	1.80
hsa-miR-27a-3p	2.07	1.59
hsa-miR-27b-3p	1.55	1.66
hsa-miR-30a-5p	1.71	4.65
hsa-miR-30b-5p	1.55	1.73
hsa-miR-30d-5p	1.67	1.98
hsa-miR-30e-3p	1.52	1.77
hsa-miR-32-5p	1.60	1.83
hsa-miR-92b-3p	1.58	1.95
hsa-miR-98-5p	1.51	1.55
hsa-miR-99a-5p	1.73	1.56
hsa-miR-105-5p	1.57	2.59
hsa-miR-125a-5p	1.67	1.72
hsa-miR-139-5p	2.35	1.91
hsa-miR-181b-5p	1.78	2.68
hsa-miR-186-5p	1.65	1.71
hsa-miR-192-5p	-2.27	-4.17
hsa-miR-193b-3p	3.33	1.53
hsa-miR-212-3p	1.81	1.67
hsa-miR-215-5p	-2.94	-4.76
hsa-miR-361-5p	1.83	1.84
hsa-miR-374a-5p	1.83	2.06
hsa-miR-450a-5p	1.59	1.65
hsa-miR-452-5p	1.61	2.58
hsa-miR-454-3p	1.59	2.44
hsa-miR-484	1.65	2.18
hsa-miR-514a-3p	1.60	3.34

miRNAs with a fold change ⩾ 1.5 in both cell lines are listed. YZSJD: Yangzheng Sanjie decoction; miRNAs: MicroRNAs.

YZSJD regulates the miRNA expression profile in GC cells

To explore the molecular mechanism involved in YZSJD-induced anticancer activity, we employed qPCR arrays to determine the changes in the miRNA expression profile in AGS and HS-746T GC cells treated with YCS. As shown in Figure 4A and B, the expression levels of a large number of miRNAs were altered. Using a recommended cut-off of 30 Ct, we identified 54 differentially expressed miRNAs in AGS cells and 60 in HS-746S cells. In both cell lines, 37 miRNAs showed the same expression alteration trend, 35 of which were up-regulated and 2 of which were down-regulated (Figure 4C and Table 2). Notably, almost all of the members of the let-7 family were altered: hsa-let-7a-5p, hsa-let-7d-3p, hsa-let-7f-5p and hsa-miR-98-5p were consistently up-regulated in both cell lines (Table 2), hsa-let-7g-5p was up-regulated only in AGS cells, and hsa-let-7b-5p, hsa-let-7c-5p, hsa-let-7d-5p, hsa-let-7e-5p and hsa-let-7i-5p were up-regulated only in HS-746T cells (data not shown).

YZSJD regulates the expression of let-7a/c-Myc in GC cells

We performed qRT-PCR to detect let-7a expression in GC cell lines with different genetic backgrounds. Significant down-regulation of let-7a expression was found in AGS, HS-746T, MKN-45 and SGC-7901 cells ($P < 0.01$) (Figure 5A). Next, the let-7a expression in AGS and HS-746T cells treated with YCS was further verified. As shown in Figure 5B, the levels of let-7a were significantly up-regulated ($P < 0.01$). We then conducted Western blot analysis to determine the c-Myc expression. The c-Myc protein expression was lower in the YZSJD groups than in the Control groups (Figure 5C and D). These results indicate that YZSJD increased the expression of let-7a and decreased c-Myc protein expression in AGS and HS-746T cells.

Abnormal expression of let-7a/c-Myc in the progression of gastric mucosa cancerization

We examined let-7a expression using ISH and detected c-Myc expression by IHC in GC tissues, matched para-carcinoma tissues and distal normal gastric mucosal tissues. A significant reduction of let-7a expression was

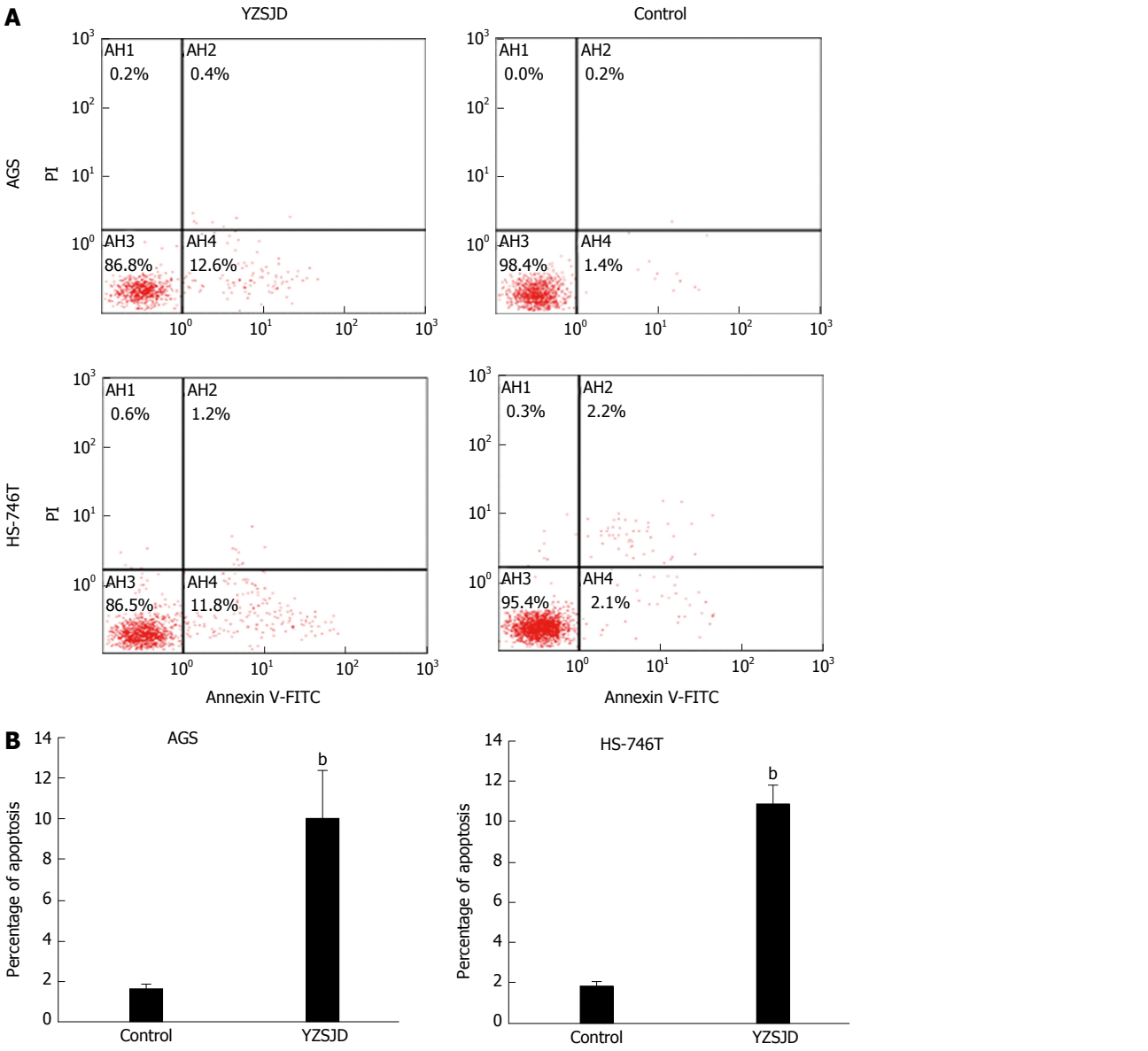

Figure 3 Effects of Yangzheng Sanjie decoction on apoptosis of gastric cancer cells. A: After 48 h treatment with 10% YCS (left) or 10% normal rat serum (right), the apoptotic cells were determined by flow cytometry. Early apoptotic cells are shown in the lower right-hand quadrant. Representative dot plots of cell apoptosis are shown. Upper: AGS; Lower: HS-746T; B: Statistical analysis of FCM data. $^{b}P < 0.01$ *vs* the control group. YCS: YZSJD-containing serum; YZSJD: Yangzheng Sanjie decoction.

observed in GC tissues, while let-7a was predominantly expressed in distal normal gastric mucosa tissues. Conversely, the IHC-based assessment of c-Myc expression demonstrated a decreasing trend from GC tissues and adjacent para-cancerous tissues to distal normal gastric tissues (Figure 6). There were significant differences in the expression of let-7a and c-Myc among the three different grades of gastric mucosa tissues ($P < 0.05$) (Table 3).

DISCUSSION

Our results show that let-7a expression was significantly lower in GC tissues than in the matched precancerous tissues and normal gastric mucosa epithelium, while c-Myc expression exhibited an opposite trend. YZSJD significantly inhibited proliferation and induced apoptosis in AGS and HS-746T cells, and YCS treatment resulted in up-regulation of let-7a and down-regulation of c-Myc in both cell types.

Currently, chemotherapy, endoscopic and surgical treatment are the major treatment modalities for GC. Since chemotherapeutics have the insurmountable problems of multi-drug resistance and negative side effects, and endoscopic and surgical treatments rely to a large extent on early detection of GC, the quality of life and 5-year overall survival rate for GC patients remain low. However, due to clear curative effects and low toxicity, traditional Chinese medicine has been recognized to have health and well-being benefits in cancer treatment[21,22].

YZSJD, a Chinese herbal compound prescribed

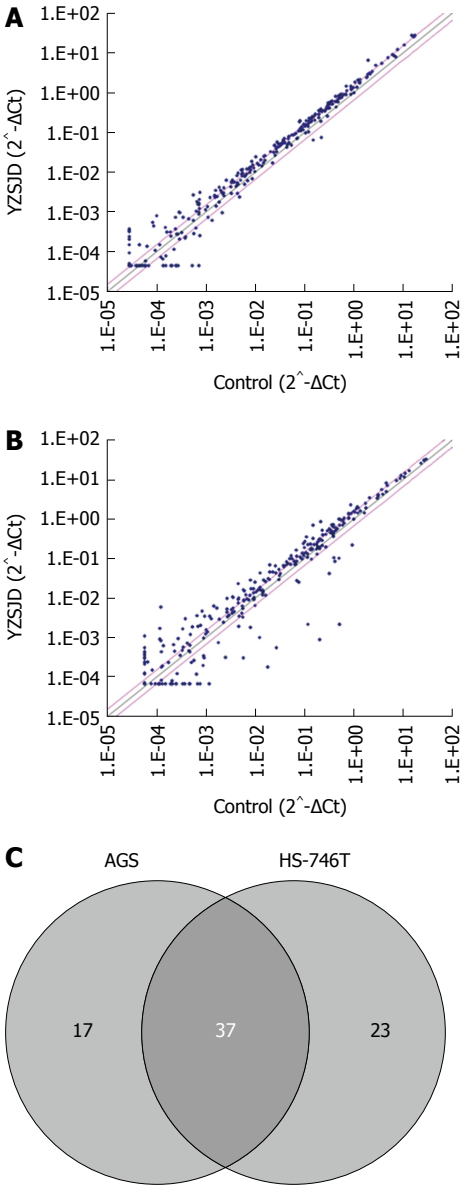

Figure 4 Effects of Yangzheng Sanjie decoction on miRNA profiles in gastric cancer cells. Scatter plot analysis of miRNA PCR array data from AGS (A) and HS-746T (B) cells treated with 10% Yangzheng Sanjie decoction-containing serum. The pink lines indicate 1.5-fold changes. C: Venn diagram analysis of differentially expressed miRNAs unique to each cell line or shared between AGS and HS-746T cells.

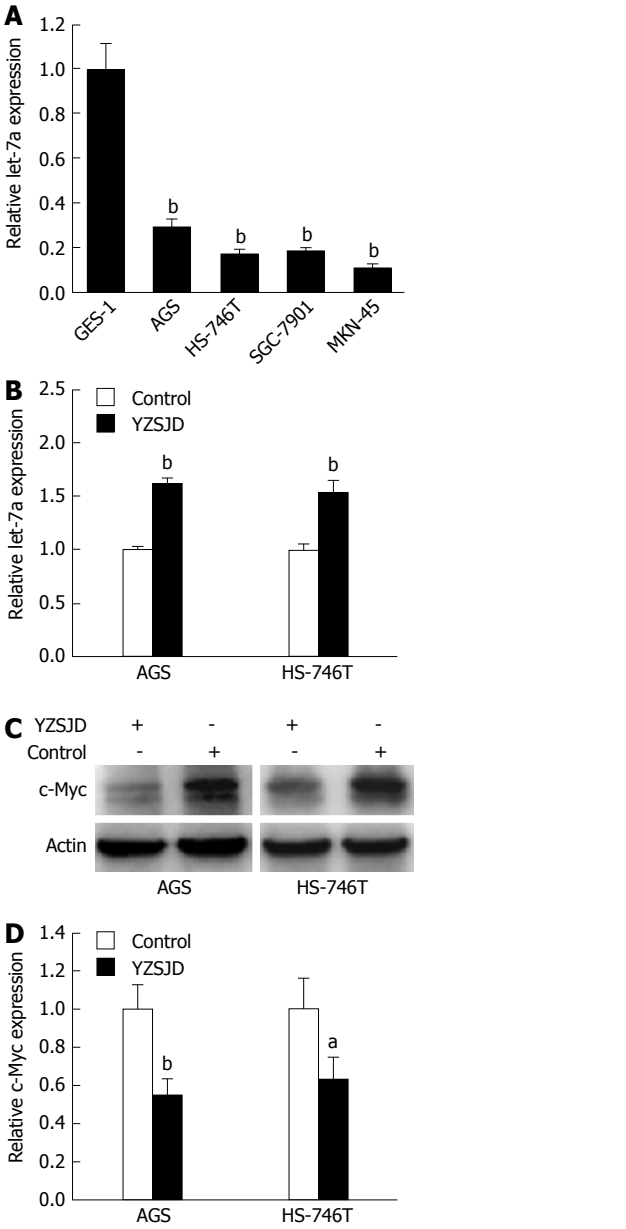

Figure 5 Effects of Yangzheng Sanjie decoction on let-7a and c-Myc expression in gastric cancer cells. A: Down-regulation of let-7a expression in GC cells; B and C: AGS and HS-746T GC cells were treated with 10% YCS or 10% normal rat serum for 48 h, and quantitative RT-PCR was performed to determine the expression levels of let-7a (B), while c-Myc expression was measured by Wester blot (C); D: Statistical analysis of Western blot data. $^{a}P < 0.05$ *vs* the control group; $^{b}P < 0.01$ *vs* the control group. YCS: YZSJD-containing serum; YZSJD: Yangzheng Sanjie decoction.

by Dr. Geng-Xin Chen, has been used to treat gastric precancerous lesions at the Second Affiliated Hospital of Guangzhou University of Chinese Medicine for more than a decade. In our previous clinical study, we found that YZSJD has a definite therapeutic effect on the precancerous lesions of patients with chronic atrophic gastritis by targeting EGF and EGFR[7,19]. In this study, we explored the anti-cancer effects of YZSJD in two GC cell lines and investigated the underlying mechanism. YZSJD significantly inhibited proliferation and induced apoptosis in AGS and HS-746T cells.

To explore the molecular mechanism involved in YZSJD-induced anticancer activity, we employed qPCR arrays to determine the changes that occurred in the miRNA expression profile in AGS and HS-746T GC cells upon YCS treatment. The expression levels of a large number of miRNAs were altered. Notably, almost all of the members of the let-7 family were up-regulated by YZSJD. Of these, hsa-let-7a-5p, hsa-let-7d-3p, hsa-let-7f-5p and hsa-miR-98-5p were consistently up-regulated in both cell lines. Because of its high abundance, we focused on let-7a in this study. Subsequent verification experiments confirmed the influence of YZSJD on let-7a expression. To monitor the downstream effectors induced by the differential

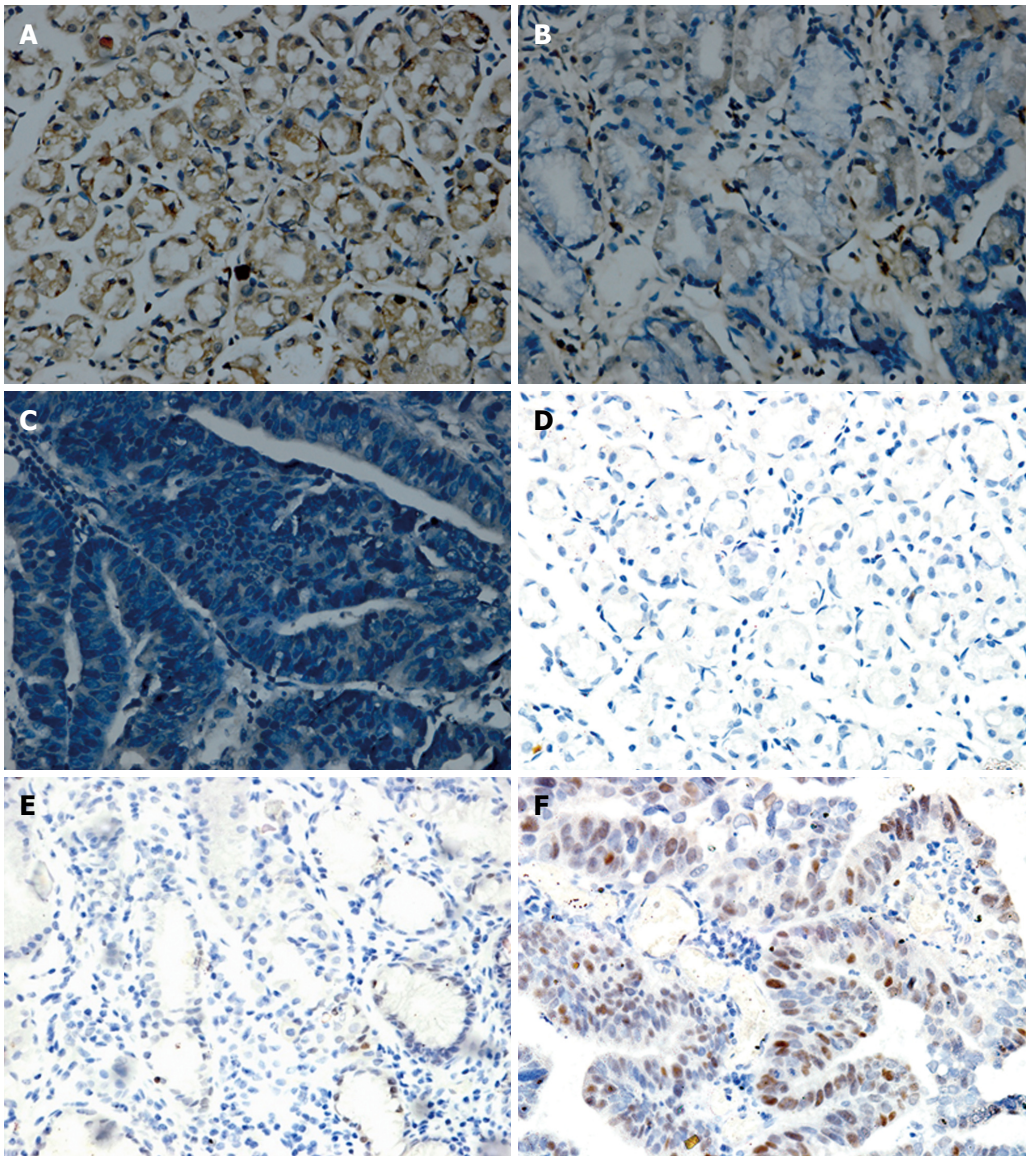

Figure 6 Expression levels of let-7a and c-Myc in gastric cancer tissues. A-C: Expression of let-7a miRNA (ISH, × 400): high in distal normal gastric tissues (A), moderate in matched para-carcinoma tissues (B) and low in GC tissues (C). D-F: Expression of c-Myc protein (IHC, × 400): low in distal normal tissues (D), moderate in adjacent tissues (E) and high in GC tissues (F). GC: Gastric cancer; ISH: *In situ* hybridization; IHC: Immunohistochemistry.

Table 3 Let-7a and c-Myc expression in gastric cancer tissue samples

Tissue	Let-7a					c-Myc				
	(-)	(+)	(++)	(+++)	*P* value	(-)	(+)	(++)	(+++)	*P* value
Ca	3	7	1	0	< 0.05	0	2	4	5	< 0.05
Pc	0	8	3	0		1	6	3	1	
N	0	1	6	4		4	6	1	0	

Staining intensity was described as negative (-), weak (+), moderate (++) and strong (+++) staining. Ca: Gastric cancer tissues; Pc: Matched para-carcinoma tissues; N: Matched distal normal gastric tissues.

expression of let-7a, we analysed the expression of c-Myc, a classical target gene of the let-7 family, and found obvious down-regulation of the protein.

Let-7 was the first human miRNA discovered and is known as a classical anti-oncomiR[23]. Pairing between let-7 and its target genes, such as Myc, RAS or HMGA2, has been shown to be pivotal in tumourigenesis[15,24,25]. Although some conflicting data have emerged indicating that let-7 may have diverse functions in different forms of cancer[26], there is evidence showing the significance of let-7 loss in GC oncogenesis and metastasis[27-30]. In this study, we further investigated the association between let-7 and the risk of GC in matched tissue samples. Consistent

with previous studies, our results indicate that let-7a expression diminished progressively during the progression of gastric mucosa cancerization.

The c-Myc oncogene is highly amplified in many cancer types and contributes to tumourigenesis[31,32]. Functioning as an important transcription factor, c-Myc protein also plays a crucial role in gastric carcinogenesis[33]. Chen *et al*[34] found that targeting c-Myc strongly inhibited cell growth and induced apoptosis in SGC7901 GC cells. Our data showed that c-Myc expression was increased markedly in GC tissues compared with matched precancerous tissues and normal gastric mucosae. Our data suggest that the reduction of let-7a may play an important role in GC occurrence and development through the derepression of c-Myc protein expression.

Several studies have revealed that let-7 inhibits the proliferation, migration, invasion and tumour metastasis of GC cells both *in vitro* and *in vivo*[28-30,35]. Let-7 miRNAs target the c-Myc 3′-UTR and negatively regulate its protein expression[15,16]. More recently, it was reported that let-7a inhibits the proliferation, migration and invasion of GC cells by suppressing the c-Myc/hnRNPA1/PKM2 pathway[18]. Given that reduced let-7a is involved in GC oncogenesis and that its expression increased in both AGS and HS-746T cells treated with YCS, we speculate that YZSJD suppressed proliferation and induced apoptosis in GC cells possibly by regulating the let-7a-c-Myc pathway.

In conclusion, the reduction of let-7a expression may play an important role in gastric tumourigenesis through c-Myc derepression. YZSJD inhibits proliferation and induces apoptosis by enhancing let-7a expression in GC cells. These findings provide new evidence that YZSJD has therapeutic potential in the treatment of GC and that miRNA expression regulation may be a novel molecular mechanism through which Chinese herbal medicine exhibits anti-cancer activity.

ACKNOWLEDGMENTS

We thank the First Affiliated Hospital of Guangzhou University of Chinese Medicine for providing human GC samples.

COMMENTS

Background

Chinese herbal medicine has become an increasingly popular cancer therapy modality. Yangzheng Sanjie decoction (YZSJD) showed beneficial effects in the treatment of precancerous lesions of gastric cancer (GC) patients according to our published data. However, its therapeutic effect and underlying mechanism associated with GC remain unknown.

Research frontiers

Let-7 miRNAs have been shown to act as tumour suppressors in GC by targeting c-Myc and are promising therapeutic targets for cancer treatment. In this study, the authors explored the let-7a-mediated anticancer effect of YZSJD *in vitro*. These data demonstrate that YZSJD inhibits proliferation and induces apoptosis by regulating the aberrant expression of let-7a and c-Myc in GC cells.

Innovations and breakthroughs

In the present manuscript, the authors present the novel findings that reduction of let-7a expression could play an important role in gastric tumourigenesis through the derepression of c-Myc expression and that YZSJD inhibits proliferation and induces apoptosis in GC cells by regulating the let-7a-c-Myc pathway.

Applications

These findings provide new evidence that YZSJD has therapeutic potential in the treatment of GC and that miRNA expression regulation may be a novel molecular mechanism through which Chinese herbal medicine exhibits anti-cancer activity, laying a new foundation for further research into the molecular mechanisms of GC treatment with Chinese herbal medicine.

Terminology

The let-7 family (let-7a/b/c/d/e/f/g/i and miR-98) is a cluster of broadly conserved miRNAs. By targeting numerous oncogenes and signalling pathways (c-Myc, Ras, HMGA2, cyclin D, cyclin A, CDK4/6, Lin28, *etc.*), let-7 blocks tumour formation, progression and metastasis and induces cell apoptosis through post-transcriptional regulation.

Peer-review

This is an interesting basic study on new traditional Chinese medicine in regulation of the proliferation and apoptosis of GC cells. The authors present the novel findings that reduction of let-7a expression could play an important role in gastric tumourigenesis through the derepression of c-Myc expression and that YZSJD inhibits proliferation and induces apoptosis in GC cells by regulating the let-7a-c-Myc pathway. These findings provide new evidence that YZSJD has therapeutic potential in the treatment of GC and that miRNA expression regulation may be a novel molecular mechanism through which Chinese herbal medicine exhibits anti-cancer activity.

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P- Reviewer: Bernal G, Eleftheriadis NP **S- Editor**: Gong ZM
L- Editor: Wang TQ **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5549-5556

DOI: 10.3748/wjg.v23.i30.5549

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

ORIGINAL ARTICLE

Case Control Study

Crohn's disease environmental factors in the developing world: A case-control study in a statewide catchment area in Brazil

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Author contributions: Zaltman C provided financial support for this work; Salgado VCL, Boechat N and Zaltman C designed the study; Salgado VCL and Zaltman C coordinated and provided the collection of all data; Leão IS, Schorr BC and Salgado VCL made the structured interviews and collected patient's data from clinical reports if needed; Salgado VCL and Luiz RR organized and analyzed the data; Nunes T, Salgado VCL and Zaltman C were involved in writing and editing the manuscript; all authors read and approved the final manuscript.

Institutional review board statement: The Study was reviewed and approved by the Comitê de Ética em Pesquisa em Seres Humanos do HUCFF/UFRJ, the Institutional Review board.

Informed consent statement: On behalf of all co-authors I declare that all study participants, or their legal guardian , provided informed written consent prior to study enrolment.

Conflict-of-interest statement: Nunes T is an employee of Nestec SA, Switzerland. The remaining authors disclose no conflicts.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: March 14, 2017
Peer-review started: March 14, 2017
First decision: April 20, 2017
Revised: May 10, 2017
Accepted: July 4, 2017
Article in press: July 4, 2017
Published online: August 14, 2017

Abstract

AIM

To identify environmental risk factors associated with the development of Crohn's disease (CD) in order to re-assess the hygiene hypothesis.

METHODS

A hospital-based, case-control study was carried out with CD patients (n = 145) and controls (n = 163)

representing a socioeconomically diverse statewide catchment area in Brazil. Controls were recruited from caregivers of patients seen in different outpatient clinics at the same hospital. A multi-item survey with 94 questions regarding family history of CD, perinatal and childhood circumstances, living conditions, tobacco use and familial socioeconomic status was carried out by interviewers.

RESULTS

On the univariate analysis, predictive variables for CD included being male, under age of 40, a high education level, urban dweller, smaller family size, exposure to enteric pathogens and user of treated water ($P < 0.005$). On the multivariate analysis, variables significantly associated with CD were male gender (OR = 2.09), under age 40 (OR = 3.10), white (OR = 2.32), from a small family in childhood (OR = 2.34) and adulthood (OR = 3.02), absence of viral infections in childhood (OR = 2.23), exposure to enteric pathogens (OR = 2.41), having had an appendectomy (OR = 2.47) and prior or current smoker (OR = 2.83/1.12).

CONCLUSION

Most variables supporting the "hygiene hypothesis" are associated with the development of CD but are not independent predictors of the diagnosis.

Key words: Crohn's disease; Environment; Hygiene hypothesis; Risk factors

Core tip: This case-control study aimed to revisit the hygiene hypothesis in inflammatory bowel disease with the inclusion of Crohn's disease (CD) patients and healthy controls representing a socioeconomically diverse statewide catchment area in Brazil. Subjects completed an extensive 94-item survey regarding perinatal and childhood circumstances, living conditions, smoking and familial socioeconomic status. Most variables supporting the hygiene hypothesis were associated with CD but were not independent predictors of the diagnosis. These findings suggest that, albeit there is an association, the influence that these variables might have on disease development is not as strong as other classic environmental factors (smoking) found to be closely related to disease onset and progression.

Salgado VCL, Luiz RR, Boechat N, Schorr BC, Leão IS, Nunes T, Zaltman C. Crohn's disease environmental factors in the developing world: A case-control study in a statewide catchment area in Brazil. *World J Gastroenterol* 2017; 23(30): 5549-5556 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5549.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5549

INTRODUCTION

The etiology of Crohn's disease (CD) remains not fully understood, being probably multifactorial, due to a complex interplay between genetic susceptibility and environmental factors[1-4]. In the United States and Europe, the rise in CD incidence was associated with the effects of industrialization and concomitant environmental and lifestyle changes[3,4]. These findings point toward the notion that environmental factors might play an important role in CD susceptibility and prevalence in the developed regions of the globe.

In this regard, the hygiene hypothesis postulates that better hygienic conditions would reduce the incidence of infections and favor the development of immune-mediated diseases[5-8]. In this hypothesis, exposure to different microbial agents could play a protective role in promoting immune system maturation by balancing pro-inflammatory Th1 response and regulatory T cell tolerance. This mechanism would provide protection against subsequent exposure to allergens and antigens and less prevalence of conditions like inflammatory bowel disease (IBD)[9]. Lack of experimental evidence, however, persists with regard to the association between the hygiene hypothesis and the increase in CD prevalence[10].

Incidence and prevalence of CD in Brazil vary according to geographical differences[11]. In the Brazilian State of Rio de Janeiro, the presence of extreme income inequality and the existence of both urban and rural areas provide an interesting case study to further understand the role of environmental factors in CD development. Specifically, the hygiene hypothesis can be tested by taking into consideration these extreme geographical differences present in this relatively small state. Therefore, the objective of the present study is to assess the environmental factors that might be associated with CD development prompted by the great inter-regional differences present in this statewide single center catchment area.

MATERIALS AND METHODS

Study design and patient inclusion

This is a case-control study including CD patients and healthy individuals. Patients were recruited at the IBD outpatient clinic of the Federal University of Rio de Janeiro (UFRJ) Hospital (HUCFF), Brazil. Healthy individuals were recruited from caregivers of patients seen in different outpatient clinics at the same hospital, with no family ties to the cases. The Ethics Committee of the Institute of Public Health Studies/UFRJ approved this study. All patients and control subjects gave written informed consent before enrolment. Data was analyzed anonymously to preserve patient's privacy

Patient selection and data collection

All included patients fulfilled the following criteria:

active follow up at the IBD outpatient clinic, established diagnosis of CD by clinical, radiological, endoscopic and histological parameters, 18 to 80 years of age, males and females. Patients and controls with established psychiatric illness or disorders that compromise the level of awareness or understanding were excluded. The sample size was based on a convenience sample according to the number of patients diagnosed with CD recorded in the HUCFF outpatient clinic.

Structured interviews were done by three interviewers trained uniformly using a prepared interview guide aiming to avoid biased information and ensuring compliance with the protocol. The 94-item questionnaire utilized in the present study was a Brazilian Portuguese translation of a previously developed Canadian questionnaire[8] with a few adaptations to the local reality. The questionnaire is mainly focused on risk factors for the development of CD as demographics aspects (sex, age, ethnicity, economic/social status, household area, family size), living conditions (housing conditions and sanitation, number of cohabitants, contact with pets and quality of water intake), smoking habits, family history (first-degree relatives), vaccinations and diseases (childhood immunizations, worms history, intestinal infections and viral diseases in childhood and appendectomy). Most of these variables were evaluated both in childhood and adulthood, before CD diagnosis. Age refers to current age at the moment the questionnaire was applied. Data was then categorized into two groups: 18 to 39 and 40 to 80 years of age.

Study subjects were racially stratified into two categories: white and non-white. Economic/social status was categorized based on the educational level and family income. Educational level was divided into three categories: elementary, high school and college degree. Family income was organized taking into consideration the household's gross monthly income according to multiples of the minimum wage: group 1 covered monthly household income of 0 to 3 minimum wages (up to about US $285 per household per month); group 2 covered household income of 3 to 5 minimum wages (US $855 to US $1425 per month) and group 3 covered increments up to more than 5 minimum wages (above US $1425 per month).

The household area was classified as urban or rural according to the Brazilian Institute of Geography and Statistics (IBGE). Definition of urban areas included cities (municipal seats), villages (district headquarters) or isolated urban areas; the areas outside of these parameters were considered rural (Ministry of Planning, Budget and Management Brazilian Institute of Geography and Statistics - IBGE 2010 Census). The assessment of housing conditions and adequate sanitation included: the presence of garbage collection, running water, and sewage drainage. Family size was characterized according to the number of inhabitants: 1 inhabitants, 2 to 3 inhabitants, and 4 to 8 inhabitants. Exposure to tobacco also was considered and three classes were defined: never (never consumed tobacco daily), previous (currently non-smokers/ex-smokers) or current tobacco user (current daily smokers) (Global Adult Tobacco Survey - GATS, 2a Edition. Atlanta, United States, 2011).

Ethical statement

The study was approved by the Ethics Committee of the University Hospital Clementino Fraga Filho of the Federal University of Rio de Janeiro, Brazil (HUCFF-UFRJ). Informed consent was obtained from all subjects prior their enrollment.

Statistical analysis

To verify differences between the two study groups (CD and controls), we used Pearson's χ^2. Univariate and multivariate logistic regression analyses were performed to identify variables associated with the development of CD. In the first multivariate analysis, all variables of interest were included in the model (analysis 1). A second multivariate analysis was then performed in which the model comprised only variables that (A) reached statistical significance or (B) had an OR higher than 2 at the first multivariate analysis (analysis 2). Significance level was set at $P \leqslant 0.05$. Statistical analyses were performed using Package for the Social Sciences (SPSS) for Windows version 17.0.

RESULTS

The study population included 308 individuals: 145 (47%) CD patients and 163 (53%) controls. Significant differences between groups were evident when analyzing variables comprising demographic characteristics, hygiene, and others environmental factors before the diagnosis as summarized in Table 1.

Univariate analysis

The variables associated with CD are shown in Table 2. In this analysis, risk factors such as being male, under 40 years old, high educational level, urban living, smaller family size in childhood, exposure to enteric pathogens and user of treated water were significantly associated with CD ($P < 0.05$).

Multivariate analysis

The logistic regression analysis was performed initially on all the variables studied (multivariate analysis 1) demonstrating that males, being under 40 years of age and white are associated with a greater likelihood to develop CD compared with controls (Table 3). Smaller families in childhood and adulthood, exposure to enteric pathogens and appendectomy prior to CD diagnosis were confirmed to be risk factors for CD development. Prior or current tobacco exposure were also identified as risk factors for developing the

Table 1 Demographics and environmental factors from Crohn's disease and control groups (*n* = 308) *n* (%)

	Groups CD[1] (*n* = 145)	Controls (*n* = 163)	*P* value[1]
Sex			
Male	62 (42.8)	43 (26.4)	0.002
Female	83 (57.2)	120 (73.6)	
Age (yr)			
18-39	79 (54.5)	50 (30.7)	< 0.0001
40-80	66 (45.5)	113 (69.3)	
Race			
White	74 (51)	65 (39.9)	0.050
Non-white	71 (49)	98 (60.1)	
Educational level			
Elementary	45 (31)	79 (48.5)	
High school	79 (54.5)	70 (42.9)	0.006
Graduate school	21 (14.5)	14 (8.6)	
Family income[2]			
< 3	46 (31.7)	54 (33.1)	
3-5	44 (30.3)	55 (33.7)	0.730
> 5	40 (27.6)	36 (22.1)	
No information	15 (10.3)	18 (11)	
Rural area			
No	114 (78.6)	106 (65)	0.008
Yes	31 (21.4)	57 (35)	
Housing conditions			
Inadequate	12 (8.3)	12 (7.4)	0.765
Adequate	133 (91.7)	151 (92.6)	
Family size in adulthood (*n*)			
Until 1	23 (15.9)	47 (28.8)	
2-3	85 (58.6)	83 (50.9)	0.024
4-8	37 (25.5)	33 (20.2)	
Family size in childhood (*n*)			
1-3	45 (31)	35 (21.5)	
4-6	61 (42.1)	56 (34.4)	0.006
> 7	39 (26.9)	72 (44.2)	
Pets			
No	30 (20.7)	21 (12.9)	0.066
Yes	115 (79.3)	142 (87.1)	
Breastfeeding			
No	12 (8.3)	13 (8)	
Yes	125 (86.2)	133 (81.6)	0.289
Unknown	8 (5.5)	17 (10.4)	
Exposure to untreated water			
No	43 (29.7)	71 (43.6)	0.012
Yes	102 (70.3)	92 (56.4)	
Vaccine (childhood)			
No	12 (8.3)	20 (12.3)	0.252
Yes	133 (91.7)	143 (87.7)	
Viral diseases (childhood)			
No	17 (11.7)	11 (6.7)	0.129
Yes	128 (88.3)	152 (93.3)	
Helmintic infections			
No	45 (31)	45 (27.6)	0.509
Yes	100(69)	118 (72.4)	
Exposure to enteric pathogens			
No	67 (46.2)	106 (65)	0.001
Yes	78 (53.8)	57 (35)	
Previous appendectomy			
No	133 (91.7)	153 (93.9)	0.466
Yes	12 (8.3)	10 (6.1)	
Tobacco exposure			
Never	86 (59.3)	106 (65)	
Prior	42 (29)	33 (20.2)	0.190
Current	17 (11.7)	24 (14.7)	
Family history			
No	132 (91)	153 (93.9)	0.346
Yes	13 (9)	10 (6.1)	

[1]χ^2 test; [2]Minimum wage. CD: Crohn's disease.

disease. Considering only variables reaching statistical significance or having an OR higher than 2 in the first multivariate analysis (multivariate analysis 2), a strong risk association was found for male sex, under age of 40, white, from smaller families in childhood and adulthood, absence of viral infections in childhood, exposure to enteric pathogens, appendectomy and prior and current smoking.

DISCUSSION

There is a slow but steady increase in CD prevalence worldwide, mainly in developing countries from Eastern Europe, Latin America and Asia, although the prevalence remains lower in comparison with Western Europe and North America[12-18]. The hygiene hypothesis has been proposed as a possible explanation for the significant increase in CD incidence in the last decades[19]. The present report assessed the hygiene hypothesis and other environmental factors in a cohort of CD patients and control subjects living in a statewide single center catchment area with great social and environmental inter-regional differences.

Considering the demographic aspects of CD, the literature presents controversial results regarding gender, although some studies have reported a slight predominance in males, which was confirmed in our study[20]. We identified a predominance of the disease in young individuals with a peak prevalence between 18 and 39 years of age, which was also comparable to previous reports[16,18,19]. The Brazilian population is characterized by a mixed race ancestry, with a genetic background originated from three main parental populations - Europeans, Brazilian Native Amerindians and Africans[21]. Interestingly, our study showed a predominance of white individuals in the CD group; this finding is in keeping with the higher incidence of IBD found in people with European ancestry.

In the univariate analysis, several important variables supporting the hygiene hypothesis were associated with CD development in the present study: access to treated water, higher educational level, smaller family size and being an urban dweller. Theoretically, the presence of these variables could be associated with less infectious diseases during the first years of life and more immune-mediated conditions at a later stage[5,6]. The relationship between access to treated water and less infections is straightforward, but the same might not be true for the other factors. In this regard, having a higher educational level or

Table 2 Univariate analysis of associations between demographics, environmental factors in Crohn's disease patients

Characteristics	Logistic univariate models		
	OR	95%CI	*P* value[2]
Sex			
Male	2.09	1.29-3.37	0.003
Female	1.00		
Age (yr)			
18-39	2.71	1.70-4.31	< 0.001
40-80	1.00		
Race			
White	1.57	1.00-2.47	0.050
Non-white	1.00		
Educational level			
Elementary	1.00		
High school	1.98	1.22-3.22	0.006
Graduate school	2.63	1.21-5.68	0.014
Family income[1]			
< 3	1.00		
3-5	0.94	0.54-1.64	0.826
> 5	1.30	0.72-2.37	0.384
No information	0.98	0.44-2.15	0.957
Rural area			
No	1.98	1.19-3.29	0.009
Yes	1.00		
Housing conditions			
Inadequate	1.14	0.49-2.61	0.765
Adequate	1.00		
Family size in adulthood (*n*)			
Until 1	1.00		
2-3	2.09	1.17-3.75	0.013
4-8	2.29	1.15-4.54	0.018
Family size in childhood (*n*)			
1-3	2.37	1.31-4.27	0.004
4-6	2.01	1.18-3.42	0.010
> 7	1.00		
Pets			
No	1.76	0.96-3.24	0.068
Yes	1.00		
Breastfeeding			
No	1.00		
Yes	1.02	0.45-2.31	0.966
Unknown	0.51	0.16-1.61	0.251
Exposure to untreated water			
No	1.00		
Yes	1.83	1.14-2.93	0.012
Vaccine (childhood)			
No	1.00		
Yes	1.55	0.73-3.29	0.254
Viral diseases (childhood)			
No	1.84	0.83-4.06	0.134
Yes	1.00		
Helmintic infections			
No	1.18	0.72-1.93	0.509
Yes	1.00		
Exposure to enteric pathogens			
No	1.00		
Yes	2.16	1.37-3.42	0.001
Previous appendectomy			
No	1.00		
Yes	1.38	0.57-3.29	0.468
Tobacco exposure			
Never	1.00		
Prior	1.57	0.92-2.60	0.101
Current	0.87	0.44-1.73	0.697
Family history			
No	1.00		
Yes	1.51	0.64-3.54	0.348

[1]Minimum wage, [2]χ^2 test. CD: Crohn's disease.

a smaller family size might function as a surrogate marker for a higher socioeconomic status, with subsequent less exposure to infectious diseases in early childhood. Likewise, being an urban dweller might lead to less exposure to livestock, decreasing the risk of parasitic infections that could modulate the immune system[10,22-24]. Despite these univariate associations supporting the hygiene hypothesis in this case-control setting, only having a smaller family size maintained statistical significance in the multivariate analysis.

Even though previous reports have indicated that pet ownership during childhood could be a risk factor for infectious diseases with subsequent reduction of immune-mediated conditions, we did not observe this association[8,14]. In fact, the association between having a pet during childhood and CD development is not entirely clear. In this regard, a Canadian case-control study has observed that contact with pets during childhood might have the opposite expected effect. In this study, pet ownership was associated with an increased risk to develop CD later on[25]. Further studies are still needed to establish a clear association between pet ownership in early childhood and an increased risk to develop CD.

The hygiene hypothesis suggests that improved sanitation and reduced exposure to enteric organisms during childhood might lead to inappropriate immunological responses later in life and higher risk of CD[26]. We observed, however, that a greater exposure to enteric pathogens was associated with a higher risk for the development of CD, a finding also confirmed by others[27]. These authors have shown that the increased risk occurs mainly in the first year after the diagnosis of infection, suggesting the existence of a detection bias. Another possible explanation for these results could be an incorrect diagnosis of intestinal infection at CD onset[27]. The same might explain the association between CD susceptibility and appendectomy. A significant association between previous appendectomy at diagnosis and the presence of CD was observed in this study. This finding, however, can be due to a misdiagnosis of appendicitis in initial cases of CD, especially in disease phenotypes with appendicular or ileocecal involvement.

The current study and several other reports have implicated smoking as a risk factor for the development of CD[28]. The pathophysiology behind the effects of smoking on CD is not well understood, but it is hypothesized that there are influences from nicotine and the participation of increased oxidative stress in the

Table 3 Multiple linear regression to assess independent factors associated with the development of Crohn's disease

Characteristics	Multivariate analysis 1			Multivariate analysis 2		
	[1]OR_{aj}	95%CI	*P* value	[1]OR_{aj}	95%CI	*P* value
Sex						
Male	2.08	1.18-3.68	0.011	2.09	1.22-3.59	0.007
Female	1.00			1.00		
Age (yr)						
18-39	2.83	1.51-5.32	0.001	3.10	1.71-5.63	< 0.001
40-80	1.00			1.00		
Race						
White	2.30	1.31-4.02	0.004	2.32	1.36-3.97	0.002
Non-white	1.00			1.00		
Educational level						
Elementary	1.00					
High school	1.44	0.75-2.75	0.266			
Graduate school	1.45	0.52-4.04	0.470			
Family income[1]						
< 3	1.00					
3-5	0.83	0.42-1.64	0.609			
> 5	0.90	0.40-2.00	0.807			
No information	1.74	0.67-4.51	0.254			
Rural area						
No	1.32	0.65-2.68	0.432			
Yes	1.00					
Housing conditions						
Inadequate	1.36	0.49-3.79	0.548			
Adequate	1.00					
Family size in adulthood (*n*)						
Until 1	1.00			1.00		
2-3	3.26	1.59-6.68	0.001	3.02	1.52-5.99	0.002
4-8	3.16	1.36-7.34	0.007	2.87	1.26-6.50	0.012
Family size in childhood (*n*)						
1-3	1.28	0.57-2.84	0.541	1.66	0.81-3.40	0.159
4-6	2.08	1.06-4.08	0.032	2.34	1.27-4.32	0.006
> 7	1.00			1.00		
Pets						
No	1.45	0.69-3.05	0.323			
Yes	1.00					
Breastfeeding						
No	1.00					
Yes	0.77	0.28-2.09	0.616			
Unknown	0.48	0.12-1.89	0.296			
Exposure to untreated water						
No	1.00					
Yes	1.54	0.80-2.93	0.188			
Vaccine (childhood)						
No	1.00					
Yes	0.57	0.20-1.58	0.284			
Viral diseases (childhood)						
No	2.42	0.90-6.48	0.078	2.23	0.88-5.62	0.088
Yes	1.00			1.00		
Helmintic infections						
No	0.91	0.51-1.65	0.776			
Yes	1.00					
Exposure to enteric pathogens						
No	1.00			1.00		
Yes	2.89	1.62-5.17	< 0.001	2.23	1.41-4.10	0.001
Previous appendectomy						
No	1.00			1.00		
Yes	2.71	0.93-7.83	0.065	2.47	0.89-6.83	0.080
Tobacco exposure						
Never	1.00			1.00		
Prior	3.13	1.57-6.26	0.001	2.83	1.46-5.47	0.002
Current	1.13	0.50-2.54	0.768	1.12	0.51-2.44	0.775
Family history						
No	1.00					
Yes	1.27	0.45-3.53	0.644			

[1]OR_{aj} = Odd Ratio adjusted. In the final model only the variables included were statistically significant (95%CI not including value 1). *P* value Hosmer-Lemeshow statistic for setting the final model = 0.406.

intestinal mucosa[29]. Smoking cessation and smoking prevention might positively influence the development and evolution of CD[30]. Interestingly, the studies on smoking are not consistent across all ethnics groups, demonstrating the potential for interactions between smoking and others environmental or genetic factors to influence disease occurrence, course or phenotype[28].

Importantly, interpretation of the present results should be performed with the perspective that there are some methodological limitations, including the possibility of reporting bias, which are common in case-controlled studies. Nonetheless, information obtained through structured questionnaires collected by graduate students, who were trained in a uniform protocol, tend to minimize this possibility. It was not possible, however, to control the recall bias, as some variables addressed issues related to early life.

Overall, in this case-control study, several factors associated with the development of CD in the univariate analysis (high education level, urban dweller, smaller family size, user of treated water) support the role of the hygiene hypothesis in the pathogenesis of CD. However, more importantly, most variables were not found to be independent predictors for the development of CD. This suggests that, albeit there is an association, the influence that these variables might have on disease development is not as strong as other classic environmental factors (smoking) found to be closely related to disease onset and progression.

COMMENTS

Background

Developing countries currently face an increase in the incidence of Crohn's disease (CD) in parallel with greater urbanization. Brazil, therefore, represents a good case study to revisit the hygiene hypothesis.

Research frontiers

The hygiene hypothesis and the contribution of each environmental factor to disease development and progression need further investigation. A better understanding of the environmental factors associated with CD can potentially have preventive or therapeutic implications.

Innovations and breakthroughs

Most case-control studies looking at environmental factors of CD took place in developed countries with a less diverse population regarding its socio-economic status and hygienic conditions. The present study used a socioeconomically diverse Brazilian population to adequately assess the influence of key factors related to the hygiene hypothesis on disease development.

Applications

These results highlight the importance of key environmental factors associated with the hygiene hypothesis but also suggest that these factors are not disease predictors, being very unlikely that they have an independent causative role.

Terminology

The hygiene hypothesis postulates that better hygienic conditions would reduce the incidence of infections and favor the development of immune-mediated diseases. In this hypothesis, exposure to different microbial agents could play a protective role in promoting immune system maturation.

Peer-review

This manuscript offers new evidence to support the hygiene hypothesis, an interesting topic which has drawn increasing attention in recent years considering this potential role in the development of CD. This case-control study was well carried out, especially the well-designed questionnaire containing most of interested environmental factors, making the data analysis reliable and convincing. The relationship between several important variables supporting the hygiene hypothesis and CD development has been clearly demonstrated in the discussion section, even taking into consideration the extreme geographical differences in Brazil. Meanwhile, the structure of this manuscript is complete and the language is perfect.

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P- Reviewer: Garcia-Olmo D, Zhu WM **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5557-5566

DOI: 10.3748/wjg.v23.i30.5557

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

ORIGINAL ARTICLE

Retrospective Cohort Study

Postoperative bleeding in patients on antithrombotic therapy after gastric endoscopic submucosal dissection

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Institutional review board statement: This research was approved by the research ethics committee in our hospital (Approval number: D1602024).

Informed consent statement: Patients were not required to provide informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: May 17, 2017
Peer-review started: May 19, 2017
First decision: June 8, 2017
Revised: June 26, 2017
Accepted: July 12, 2017
Article in press: July 12, 2017
Published online: August 14, 2017

Abstract

AIM

To investigated the relationship between postoperative bleeding following gastric endoscopic submucosal dissection (ESD) and individual antithrombotic agents.

METHODS

A total of 2488 gastric neoplasms in 2148 consecutive patients treated between May 2001 and June 2016 were studied. The antithrombotic agents were categorized into antiplatelet agents, anticoagulants, and other antithrombotic agents, and we included combination therapies [*e.g.*, dual antiplatelet therapy (DAPT)]. The risk factors associated with post-ESD bleeding, namely, antithrombotic agents overall, individual antithrombotic agents, withdrawal or continuation of antithrombotic agents, and bleeding onset period (during the first six days or thereafter), were analyzed using univariate and multivariate analyses.

RESULTS

The en bloc resection and complete curative resection rates were 99.2% and 91.9%, respectively. Postoperative bleeding occurred in 5.1% cases. Bleeding occurred in 10.3% of the patients administered antithrombotic agents. Being male (P = 0.007), specimen size (P < 0.001), and antithrombotic agent used (P < 0.001) were independent risk factors for postoperative bleeding. Heparin bridging therapy (HBT) (P = 0.002) and DAPT/multidrug combinations (P < 0.001) were independent risk factors associated with postoperative bleeding. The bleeding rate of the antithrombotic agent continuation group was significantly higher than that of the withdrawal group (P < 0.01). Bleeding within postoperative day (POD) 6 was significantly higher in warfarin (P = 0.015), and bleeding after POD 7 was significantly higher in DAPT/multidrug combinations (P = 0.007). No thromboembolic events were reported.

CONCLUSION

We must closely monitor patients administered HBT and DAPT/multidrug combinations after gastric ESD, particularly those administered multidrug combinations after discharge.

Key words: Gastric cancer; Endoscopic submucosal dissection; Postoperative hemorrhages; Antithrombotic agent; Heparin

Core tip: The major complication of gastric endoscopic submucosal dissection (ESD) is postoperative bleeding. Previous studies reported the relationship between postoperative bleeding and antithrombotic agents. We aimed to investigate postoperative bleeding following gastric ESD in relation to specific antithrombotic agents. We showed that antithrombotic agents, in particular heparin bridging therapy and dual antiplatelet therapy/multidrug combination, were independent risk factors for delayed bleeding. Furthermore, bleeding in the early period was significantly higher for warfarin, and bleeding in the late period was significantly higher for multidrug combination. We must strictly observe multidrug combination users especially after discharge.

Sato C, Hirasawa K, Koh R, Ikeda R, Fukuchi T, Kobayashi R, Kaneko H, Makazu M, Maeda S. Postoperative bleeding in patients on antithrombotic therapy after gastric endoscopic submucosal dissection. *World J Gastroenterol* 2017; 23(30): 5557-5566 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5557.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5557

INTRODUCTION

Endoscopic submucosal dissection (ESD) for early gastric cancer is routinely performed worldwide, because it is minimally invasive and effective[1-3]. The most common complication associated with gastric ESD is postoperative bleeding, which has a frequency of 3.1%-6.5%[4-6].

The number of individuals administered antithrombotic agents has increased over recent years. The Japan Gastrointestinal Endoscopy Society (JGES) guidelines indicate that withdrawal or continuation of antithrombotic agents depends on whether the patient is at a high or low risk of thromboembolism during ESD[7]. The risk of postoperative bleeding increases when gastric ESD is performed on patients on antithrombotic agent therapy[8-10], and antithrombotic agent therapy is an independent risk factor for postoperative bleeding[11]. Heparin bridging therapy (HBT) is recommended for patients who are on anticoagulant therapy and are at a high risk of thromboembolism[7], and postoperative bleeding associated with gastric ESD worsens with HBT[12-14]. Furthermore, the preventive effect of HBT on thromboembolism is low[15]. By contrast, the risk of thromboembolism following antithrombotic agent withdrawal is 0.6%-4.2%[16-18]. Thus, caution is required when deciding whether to withdraw or continue antithrombotic agents in preparation for endoscopic treatment.

Few studies have investigated the incidence of thromboembolism following gastric ESD in relation to the different types of antithrombotic agent. Moreover, the safety and validity of HBT remain controversial. This study aimed to evaluate postoperative bleeding, the washout periods, and the thromboembolism incidence in relation to different antithrombotic agents following gastric ESD performed on patients administered antithrombotic agents.

MATERIALS AND METHODS

Between May 2001 and June 2016, ESD was performed on 2148 patients and 2488 early gastric cancer lesions. Of these patients, 50 with cancer in their remnant stomachs and four with gastric tubes were excluded; thus, 2094 patients and 2434 lesions were evaluated. Multiple lesions resected en bloc were included in this study, and since the analysis was based on ESD-induced ulcers, 2094 patients and 2378 ulcers were investigated. We retrospectively reviewed the clinical records of all patients after obtaining approval from the institutional review board.

ESD is indicated for differentiated mucosal cancer lesions without ulcers (UL[-]), regardless of size, differentiated mucosal cancer lesions ≤ 3 cm with ulcers (UL[+]), UL[-] undifferentiated mucosal cancer ≤ 2 cm in size[19-21], and for lymph node metastasis-free and distant metastasis-free lesions that have been confirmed by preoperative computed tomography.

The antithrombotic agents were categorized into antiplatelet agents (low-dose aspirin and thienopyridine), anticoagulants [warfarin and direct oral anticoagulants (DOAC)], and other antithrombotic

agents, and we included combination therapies, for example, dual antiplatelet therapy (DAPT). Of the patients who received HBT, 94.9% were switched from warfarin; therefore, the warfarin group was called the non-HBT warfarin monotherapy group and it was analyzed separately from the group administered HBT.

The risk factors associated with postoperative bleeding were investigated in relation to age, sex, the size of the specimen, the tumor's morphology, the tumor's depth, the presence or absence of ulcerative findings, antithrombotic agent use, and the treatment outcomes. The postoperative bleeding risk was investigated in relation to the aforementioned drug categories. The time to bleeding with respect to each drug was separated into an early period, that is, before postoperative day (POD) 6, and a late period, that is, after POD 7. The withdrawal and continuation of the antithrombotic agents, including HBT, were investigated.

Management and procedure

If a patient was taking an oral antithrombotic agent, we consulted the attending physician before the procedure began and determined the feasibility of treatment withdrawal and the duration of the withdrawal period using the JGES guidelines. If patients were taking DAPT or multiple antithrombotic agents, we changed their treatment to low-dose aspirin monotherapy following preprocedural consultations with their attending physicians.

The patients in the antithrombotic agent continuation and withdrawal groups were hospitalized the day before the ESD. Patients switched to HBT from anticoagulant therapy were hospitalized three days before the ESD, and continuous intravenous drip infusions of unfractionated heparin were initiated. In the HBT group, we adjusted the dose to increase the activated partial thromboplastin time to 1.5-2-times that of the pretreatment value. We confirmed that the prothrombin time (PT)-international normalized ratio (INR) was not excessively prolonged on the day of the ESD. We interrupted the HBT 6 h before the ESD, and after the ESD, we confirmed adequate hemostasis at the ulcer's base and restarted the HBT 3 h later. Anticoagulant therapy was reinstated from POD 2 following second-look endoscopy (SLE) on POD 1 to confirm hemostasis. To reach the peak plasma concentrations rapidly, the patients received twice their usual doses of anticoagulant therapy on POD 2, 1.5-times their doses on POD 3, and their usual doses from POD 4 onwards. In the HBT group, we stopped treatment after confirming that the PT-INR had reached its optimum range following the reinstatement of oral treatment. We also reinstated oral treatment in the withdrawal group from POD 2 onwards following SLE. All of the patients received omeprazole drip infusions (40 mg/d) on POD 1. From POD 2 onwards, they received an oral proton pump inhibitor (PPI) that, until 2014, comprised omeprazole (20 mg/d) and after 2015, comprised esomeprazole (20 mg/d); this treatment continued for at least eight weeks after ESD.

The gastric ESDs were performed using a conventional procedure[11,22]. After making the incision, a coagulation procedure was performed on the exposed blood vessels that remained in the ulcer's base using hemostatic forceps (Coagrasper; Olympus Medical Systems Corporation, Tokyo, Japan). A mixture containing aluminum hydroxide gel, liquid magnesium hydroxide, and 10000 U thrombin (approximately 100 mL) was dispersed.

SLE was performed on all patients on POD 1 to check the bleeding from the ulcers' bases, and if exposed blood vessels were detected, prophylactic hemostasis using a clip or coagulation hemostasis was performed. After confirming hemostasis, fluid intakes were resumed, and liquid meals were reinstated from POD 2. Solid meals, oral antithrombotic agents, and PPIs were reinstated simultaneously.

Postoperative bleeding definitions

Postoperative bleeding was defined as clinical evidence of bleeding in ESD-induced ulcers, which included the occurrence of overt hematemesis, the presence of melena, the presence of blood or clots in the stomach, spots of bleeding observed endoscopically, or a reduction in the hemoglobin level of > 2 g/dL. Preventive hemostasis of visible vessels without evidence of bleeding during SLE was not included in the analysis. Most patients were hospitalized for six days after the ESD; hence, bleeding that occurred during hospitalization was defined as early-phase postoperative bleeding, and that which occurred after discharge was defined as late-phase postoperative bleeding.

Statistical analysis

Some of the patients had more than one gastric neoplasm and underwent one or more ESDs. For the statistical analyses, the data from different ESD-induced ulcers were considered to represent statistically independent observations. The patients' characteristics are expressed as the means and the standard deviations. The groups' mean quantitative values were compared using analyses of variance followed by *t* tests. To investigate the potential risk factors associated with post-ESD bleeding, we analyzed the following variables: age; sex; the use of antithrombotic agents, including aspirin, thienopyridine, warfarin, DOACs, HBT, other antithrombotic agents, and DAPT/multidrug combinations; the resected specimen's maximum diameter; the tumor's location; pathological factors, including the macroscopic type, histological depth, and lymphovascular invasion; the ulcer's characteristics; the procedure time; complications, including perforations, postoperative perforations, postoperative bleeding, and thromboembolism; drug withdrawal or

Table 1 Clinicopathological features and treatment outcomes of all 2094 patients (2434 lesions and 2378 ulcers) *n* (%)

Age (mean ± SD, yr)	72 ± 6.9
Gender	
Male	1786 (75.1)
Female	592 (24.9)
Location	
U	412 (17.5)
M	742 (31.2)
L	122 (51.5)
Morphology	
Protruded	1042 (43.8)
Flat/depressed	1336 (56.2)
Specimen size, (mean ± SD, mm)	39 ± 9.8
Depth of invasion	
M	2227 (93.7)
SM	151 (6.3)
Ulcerative findings	
(+)	203 (8.5)
(-)	2175 (91.5)
Anticoagulant agents	
(+)	447 (18.8)
(-)	1931 (81.2)
En-bloc resection	99.2%
R0+curative resection	91.9%
Mean procedure time ± SD (min)	49 ± 30.1
Complications	
Perforation	74 (3.1)
Delayed perforation	2 (0.8)
Delayed bleeding	122 (5.1)
Thromboembolism	0

continuation; and the bleeding time frame. For the univariate analyses, the categorical variables were compared using the χ^2 test and Fisher's exact test, as appropriate, followed by Fisher's least significant difference post hoc test, and those variables with P values < 0.05 were included in the multivariate analyses. The ORs and 95%CIs were calculated using logistic regression analyses to identify the factors associated with postoperative bleeding. P values < 0.05 were considered statistically significant. All of the statistical analyses were conducted using SPSS, version 13.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

The ESD procedures were well tolerated by the patients, and their cardiac and respiratory parameters remained stable throughout the procedures. Table 1 presents the clinicopathological characteristics of and the treatment outcomes from the 2094 patients (2434 lesions and 2378 ulcers).

The en-bloc resection and complete curative resection rates were 99.2% and 91.9%, respectively. There were 74 (3.1%) cases with perforations, two (0.08%) postoperative perforations, and 122 (5.1%) cases of postoperative bleeding. No thromboembolic events occurred.

Risk factors associated with postoperative bleeding

To investigate the risk factors associated with postoperative bleeding, the ulcers (n = 2378) were divided into a bleeding group (n = 122) and a non-bleeding group (n = 2256). Overall, 447 ulcers (18.8%) occurred in the patients administered antithrombotic agents, and the rate of bleeding was 10.3% (46/447). The univariate analysis showed that being male (P = 0.002), a large specimen (P < 0.001), submucosal invasive cancer (P = 0.045), and antithrombotic agent use (P < 0.001) were significantly associated with post-ESD bleeding (Table 2). The multivariate analysis revealed that being male (OR = 2.103, 95%CI: 1.224-3.611, P = 0.007), the specimen size (OR = 1.025, 95%CI: 1.013-1.037, P < 0.001), and antithrombotic agent use (OR = 2.643, 95%CI: 1.796-3.889, P < 0.001) were independent risk factors for postoperative bleeding (Table 3).

Risk factors for post-ESD bleeding according to the drug(s) administered

The antithrombotic agent group comprised 447 ulcers, and postoperative bleeding occurred in 211 (47.2%) cases on low-dose aspirin, 19 (4.3%) cases on thienopyridine, 17 (3.8%) cases on warfarin, 18 (4.0%) cases on DOACs, 70 (15.7%) cases on other antithrombotic monotherapies, 39 (8.7%) cases on HBT, and 75 (16.8%) cases on DAPT/multidrug combinations (Table 4). In addition, 94.9% (37 cases) of the HBT cases were warfarin users who were switched to HBT.

The bleeding rates were 5.7% (12/211 cases, P = 0.224) in the low-dose aspirin group, 0% (0/19 cases, P = 0.379) in the thienopyridine group, 5.9% (1/17 cases, P = 0.498) in the warfarin group, 5.6% (1/18 cases, P = 0.725) in the DOAC group, 4.3% (3/70 cases, P = 0.883) in the other antithrombotic monotherapy group, 15.4% (6/39 cases, P < 0.01) in the HBT group, and 30.7% (23/75 cases, P < 0.01) in the DAPT/multidrug combination group. The multivariate analysis determined that HBT and DAPT/multidrug combinations were independent risk factors for post-ESD bleeding [HBT: OR = 4.244, 95%CI: (1.736-10.380), P = 0.002; DAPT/multidrug combinations: OR = 10.325, 95%CI: (6.060-17.593), P < 0.001] (Table 5).

Rates of bleeding associated with antithrombotic agent withdrawal or continuation

The postoperative bleeding rates in the control (1931 ulcers) and withdrawal (401 ulcers) groups were 3.9% (76/1931 ulcers) and 8.0% (32/401 ulcers), respectively, a difference that was significant (P < 0.01) (Table 6). The postoperative bleeding rates in the withdrawal (401 ulcers) and continuation (46 ulcers) groups were 8.0% (32/401 ulcers) and 30.4% (14/46 ulcers), respectively, a difference that was significant (P < 0.01) (Table 6).

Bleeding times according to the drug(s) administered

Bleeding during the early period (up to POD 6) was common among the patients administered warfarin (P

Table 2 Univariate analysis of risk factors of delayed bleeding *n* (%)

	n = 2378	Delayed bleeding		*P* value	OR	95%CI
		(+) *n* = 122	(-) *n* = 2256			
Age (mean ± SD, yr)		71.5 ± 8.7	71.1 ± 8.8	0.622[2]	1.005	0.984-1.027
Gender						
Male	1786 (75.1)	106 (5.9)	1680 (94.1)	0.002[1]	2.271	1.331-3.875
Female	24.9 (592)	16 (2.7)	576 (97.3)			
Location						
U	412 (17.3)	17 (4.1)	395 (95.9)		Reference	1.0
M	742 (31.2)	40 (5.4)	702 (94.6)	0.342	1.303	0.755-2.250
L	1224 (51.5)	65 (5.3)	1159 (94.7)	0.344	1.324	0.741-2.366
Morphology						
Protruded	1042 (43.8)	49 (4.7)	993 (95.3)	0.404[1]	0.854	0.589-1.238
Flat/depressed	1336 (56.2)	73 (5.5)	1263 (94.5)			
Specimen size (mean ± SD, mm)	38.9 ± 2.2	44.4 ± 15.1	38.9 ± 13.1	< 0.001[2]	1.025	1.014-1.037-
Depth of invasion						
M	2227 (93.7)	109 (4.9)	2118 (95.1)	0.045[1]	1.830	1.004-3.336
SM	151 (6.3)	13 (8.6)	138 (91.4)			
Ulcerative findings						
(+)	203 (8.5)	12 (5.9)	191 (94.1)	0.598[1]	1.179	0.638-2.179
(-)	2175 (91.5)	110 (5.1)	2065 (94.9)			
Anticoagulant agents						
(+)	447 (18.8)	46 (10.3)	401 (89.7)	< 0.001[1]	2.800	1.911-4.101
(-)	1931 (81.2)	76 (3.9)	1855 (96.1)			
En-bloc resection rate	99.2%	99.1%	99.2%	0.979	0.973	0.129-7.329
R0+curative resection rate	91.9%	95.1%	97.1%	0.198	1.744	0.740-4.017
Median procedure time (min)	49 ± 30.1	50 ± 30.8	50 ± 32.2	0.885[2]	1.033	0.840-2.354

[1]χ^2 test; [2]*t*-test.

Table 3 Multivariate analysis of risk factors of delayed bleeding

	P value	OR	95%CI
Male	0.007	2.103	1.224-3.611
Median specimen size	< 0.001	1.025	1.013-1.037
SM	0.187	1.516	0.817-2.812
Anticoagulant agents (+)	< 0.001	2.643	1.796-3.889

= 0.015), and bleeding during the late period (from POD 7 onwards) was common among the patients administered DAPT/multidrug combinations (*P* = 0.007) (Table 7). Bleeding was commonly observed during the early period in patients administered HBT, a difference that was not significant compared with the other treatments.

DISCUSSION

In this study, we investigated the risk of bleeding associated with gastric ESD, the bleeding time, and the risk of bleeding associated with treatment withdrawal or continuation according to the antithrombotic agent(s) administered. We determined that being male, a large specimen, and antithrombotic agent use were independent risk factors for postoperative bleeding. Previous reports revealed that the prevalence of ischemic heart disease and stroke is higher for male than for female[23-25]. Furthermore, since ischemic heart disease and stroke are closely related to antithrombotic therapy, we considered that male sex is an independent risk factor.

Furthermore, HBT and DAPT/multidrug combinations were independent risk factors for postoperative bleeding. This study's findings showed that postoperative bleeding was significantly higher in the group that continued antithrombotic agents compared with the group that withdrew antithrombotic agents. Our findings also showed that early-phase bleeding was more frequent in association with HBT and that late-phase bleeding was more frequent in association with DAPT/multidrug combinations.

The bleeding rate for patients on antithrombotic agents is high at 23.3%-35.5%[26,27], that associated with HBT is higher at 23.8%-61.5%, and it is even higher in association with multidrug combinations[12-14,28,29]. We found that HBT and DAPT/multidrug combinations were independent risk factors associated with postoperative bleeding, which concurs with previous reports. However, until now, the risk of post-ESD bleeding and the bleeding time frames in the context of individual antithrombotic agents had not been investigated in a large number of subjects. Furthermore, this is the first study to compare the risk of bleeding in a control group and a treatment withdrawal group, and in a withdrawal group and treatment continuation group.

In this investigation, 94.9% (37/39) of the patients treated with HBT were switched from warfarin therapy. When HBT patients are administered warfarin, they will be affected by the HBT; hence, the effect of warfarin alone on postoperative bleeding cannot be analyzed. Consequently, this study's analysis involved assigning the patients to an HBT group or a non-HBT warfarin

Table 4 Univariate analysis of risk factors for delayed bleeding by each antithrombotic agent *n* (%)

	Delayed bleeding		*P* value[1]	OR	95%CI
	(+)	(-)			
Aspirin					
(+) (*n* = 211)	12 (5.7)	199 (94.3)	0.224	1.472	0.787-2.753
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
Thienopyridine					
(+) (*n* = 19)	0 (0)	19 (100)	0.379	0.990	0.985-0.994
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
Warfarin					
(+) (*n* = 17)	1 (5.9)	16 (94.1)	0.498	1.525	0.200-11.653
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
DOAC					
(+) (*n* = 18)	1 (5.6)	17 (94.4)	0.725	1.436	0.189-10.930
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
Others					
(+) (*n* = 70)	3 (4.3)	67 (95.7)	0.883	1.093	0.336-3.554
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
HBT					
(+) (*n* = 39)	6 (15.4)	33 (84.6)	< 0.01	4.438	1.805-10.911
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			
DAPT/multidrug combination					
(+) (*n* = 75)	23 (30.7)	52 (69.3)	< 0.01	10.796	6.280-18.558
(-) (*n* = 1931)	76 (3.9)	1855 (96.1)			

[1]χ^2 test. DOAC: Direct oral anticoagulants; HBT: Heparin bridging therapy; DAPT: Dual antiplatelet therapy.

Table 5 Multivariate analysis of risk factors for delayed bleeding by each antithrombotic agent

	P value	OR	95%CI
HBT	0.002	4.244	1.736-10.380
DAPT/multidrug combination	< 0.001	10.325	6.060-17.593

HBT: Heparin bridging therapy; DAPT: Dual antiplatelet therapy.

monotherapy group. In the warfarin group, 94% (16/17) of the patients continued their treatment, but there was no significant difference in relation to bleeding, a finding that has never been reported before.

The postoperative bleeding rate was high in the antithrombotic agent continuation group compared with the control and withdrawal groups. Therefore, to reduce the bleeding risk, antithrombotic agent withdrawal is preferable. However, between 1% and 4.2% of patients at a high risk of thromboembolism develop thromboembolisms following drug withdrawal[15-18]. Thus, drug withdrawal may trigger serious life-threatening complications. In contrast, another report states that the prognosis is significantly worse following the onset of gastrointestinal bleeding after percutaneous coronary intervention[30]. Regarding therapy withdrawal or continuation, physicians should examine a patient's thromboembolism risk and the drug type, and consider implementing tailor-made treatment for each case.

The JGES guidelines recommend that for patients at a high risk of thromboembolism during endoscopic procedures that are associated with a high risk of bleeding, including ESD, it is preferable to administer aspirin alone with no treatment withdrawal. The guidelines from the United States and Europe also recommend continuing aspirin therapy[7,31,32]. Therefore, the continuation of aspirin therapy was permitted in this study. However, the times at which drug treatments other than aspirin are resumed in DAPT and combination therapy regimens to reduce the risk of bleeding must be investigated. On the other hand, withdrawing anticoagulants, for example, warfarin, will likely induce serious thromboembolism. Therefore, HBT after withdrawal is recommended for endoscopic procedures[7]. Patients administered warfarin and periprocedural HBT are at a higher risk of bleeding compared with those who are not administered HBT[33,34]. Currently, insufficient evidence exists that supports the prevention of thromboembolism by HBT, and reports have been published that state that it either has no effect on or it has an equivalent efficacy at preventing arterial thromboembolism and reducing the risk of bleeding[15,33]. In this study, perioperative thromboembolism did not occur in any of the patients; hence, the prophylactic effect of HBT on blood clots could not be verified. This study's results demonstrated that HBT is an independent risk factor associated with bleeding, and considering previous reports, we recommend that patients on warfarin should either be switched to DOAC rather than HBT, or that they should continue warfarin treatment when ESD is indicated.

The times at which HBT and antithrombotic agent treatments are reinstated are determined once hemostasis has been confirmed; however, definitive timings have not been established. We reinstated heparin treatment from 3 h after ESD, after performing

Table 6 Investigation of rate of bleeding based on withdrawal or continuation of antithrombotic agent *n* (%)

	Delayed bleeding		*P* value[1]	OR	95%CI
	(+)	(-)			
Control group/withdrawal group					
Control (*n* = 1931)	76 (3.9)	1855 (96.1)	< 0.01	0.472	0.308-0.725
Withdrawal (*n* = 401)	32 (8.0)	369 (92.0)			
Withdrawal group/continuation group					
Withdrawal (*n* = 401)	32 (8.0)	369 (92.0)	< 0.01	5.045	2.445-10.411
Continuation (*n* = 46)	14 (30.4)	32 (69.6)			

[1]χ^2 test.

Table 7 Investigation of bleeding time by each drug

	Bleeding (+) *n* = 46		*P* value[1]	OR	95%CI
	Early phase	Late phase			
Aspirin (*n* = 12)	6	6	0.477	0.619	0.164-2.332
Thienopyridine (*n* = 0)	0	0	-	-	-
Warfarin (*n* = 7)	6	1	0.015	0.083	0.009-0.767
DOAC (*n* = 1)	0	1	1.000	1.038	0.964-1.118
Others (*n* = 3)	2	1	0.561	0.327	0.027-3.892
HBT (*n* = 6)	5	1	0.068	0.108	0.011-1.015
DAPT/multidrug combination (*n* = 23)	5	18	0.007	5.600	1.530-20.492

[1]Fisher's exact test. DOAC: Direct oral anticoagulants; HBT: Heparin bridging therapy; DAPT: Dual antiplatelet therapy.

adequate hemostasis of the ulcer base and accounting for the thromboembolism risk. Furthermore, we performed SLE on the day after ESD, and we performed preventive hemostasis on the exposed blood vessels in the ulcers' bases, even if there was no bleeding. Early bleeding was common before POD 6 in the HBT group. This may have been caused by the synergistic pharmacological effects of the heparin and the anticoagulants, which were reinstated from POD 2 onwards, reaching their peak plasma concentrations. In contrast, in the DAPT/multidrug combination group, the plasma concentrations of the respective antithrombotic agents were stable and required time to reach their peak ranges, and late bleeding became common from POD 7 onwards.

Many reports describe post-ESD bleeding prevention, and coagulation immediately after ESD is commonly used and is effective[5]. Applying a polyglycolic acid sheet to and spreading fibrin glue on ESD-induced ulcer bases are novel, easy, and effective approaches to the management of post-ESD bleeding in patients on antithrombotic agents[35-37], and they will be useful for patients who are at a high risk of bleeding.

The findings from a multicenter prospective randomized study of SLE undertaken by Mochizuki *et al*[38] showed that the postoperative bleeding rates did not differ significantly between the SLE and the non-SLE groups. These investigators reported that scheduled SLE could not be expected to reduce bleeding. However, another report states that SLE and third-look esophagogastroduodenoscopy are useful for preventing post-ESD bleeding in patients on antithrombotic agents[39]. Further investigations into these approaches are warranted.

The limitations of this study are as follows. First, this is a single-center retrospective cohort study. Second, the relationship between *Helicobacter pylori* (*H. pylori*) and postoperative bleeding after gastric ESD was not investigated. We could not confirm the information about *H. pylori* infection and eradication, particularly in the initial cases, because of the long research period. As a result, only about 60% of the total information of *H. pylori* could be obtained; thus, the state of *H. pylori* infection was excluded as a factor for postoperative bleeding in this study.

This study's findings demonstrated that HBT and DAPT/multidrug combinations are independent risk factors for postoperative bleeding in patients on antithrombotic agents who undergo gastric ESD. Adequate management that considers the prevention of bleeding and thromboembolism is important.

What is already known on this topic

Antithrombotic agent therapy increases the risk of bleeding after ESD. Serious thromboembolism may occur following antithrombotic agent withdrawal.

What this study adds to our knowledge

HBT and DAPT/multidrug combinations are independent risk factors for bleeding post-ESD. Early-phase bleeding was more frequently associated with HBT, and late-phase bleeding was more frequently associated with DAPT/multidrug combinations. When patients on warfarin monotherapy and those who are switched from warfarin to HBT are investigated separately, HBT alone becomes a risk factor.

COMMENTS

Background

The most common complication associated with gastric endoscopic submucosal dissection (ESD) is postoperative bleeding. An increasing number of reports have revealed that antithrombotic agent therapy is a risk factor after ESD. Withdrawal or continuation of antithrombotic agents is decided depending on the patient's risk of thromboembolism. However, many reports showed that antithrombotic agents promote the risk of postoperative bleeding and withdrawal promotes thrombosis. Although it is clinically questionable whether all types of antithrombotic agents can be equally treated, detailed investigation has not yet been performed and the risk and benefit of continuation or withdrawal remain controversial. Thus, this study aimed to evaluate postoperative bleeding, the washout periods, and the thromboembolism incidence in relation to different antithrombotic agents following gastric ESD performed on patients administered antithrombotic agents.

Research frontiers

A previous retrospective study showed that the bleeding rate for gastric ESD patients on antithrombotic agents is high at 23.3%-35.5%, that associated with heparin bridge therapy (HBT) is higher, and it is even higher in association with multidrug combinations. The Japan Gastrointestinal Endoscopy Society (JGES) guidelines indicate that withdrawal or continuation of antithrombotic agents depends on whether the patient is at a high or low risk of thromboembolism during ESD. The JGES guidelines recommend that for patients at a high risk of thromboembolism during ESD, administration of aspirin alone with no treatment withdrawal is preferred, and the guidelines from the United States and Europe also recommend continuing aspirin therapy. Although HBT after withdrawal is also recommended for endoscopic procedures in the JGES guidelines, patients administered warfarin and periprocedural HBT are reported at a higher risk of bleeding compared with those who are not administered HBT. Furthermore, only few reports are available on the relationship with direct oral anticoagulants or dual antiplatelet therapy (DAPT). Studies on drug continuation or withdrawal, by each drug, and the period to bleeding also remain unreported.

Innovations and breakthroughs

The authors determined that being male, a large specimen, and antithrombotic agent use were independent risk factors for postoperative bleeding; this finding is almost the same as previously reported. Furthermore, HBT and DAPT/multidrug combinations were independent risk factors for postoperative bleeding. These study's findings showed that postoperative bleeding was significantly higher in the group that continued antithrombotic agents compared with the group that withdrew antithrombotic agents. These findings also showed that early-phase bleeding was more frequent in association with HBT and that late-phase bleeding was more frequent in association with DAPT/multidrug combinations. The authors recommend paying strict attention to observe multidrug combination users, especially after discharge. However, until now, the risk of post-ESD bleeding and the bleeding time frames in the context of individual antithrombotic agents have not been investigated in a large number of subjects. The present study is the first to compare the risk of bleeding in a control group and a treatment withdrawal group, and in a withdrawal group and a treatment continuation group.

Applications

The results of this study serve as additional evidence to support the calculation of the risk of postoperative bleeding after gastric ESD posed by each antithrombotic agent based on continuation or withdrawal of the antithrombotic agent. Based on the present research, more robust evidence can be obtained in a prospective study.

Terminology

ESD is one of the endoscopic treatment procedures for superficial gastrointestinal cancer, which can be resected in en-bloc manner.

Peer-review

This original research paper investigates retrospectively postoperative bleeding in patients on antithrombotic therapy after gastric ESD from the clinical records of patients in a large cohort. The authors showed that antithrombotic agent in particular heparin bridging therapy and DAPT/multidrug combination were independent risk factors for delayed bleeding, and furthermore, bleeding in the early period was significantly higher for warfarin, and bleeding in the late period was significantly higher for multidrug combination. They recommend to pay attention strictly to observe multidrug combination users especially after discharge. The structure of the manuscript is complete. The scientific question and the aim of the study was stressed clearly in the introduction. The study is well-designed, and all methods and techniques were explained in details. The results add new findings and information to current knowledge. The results were discussed comprehensively.

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P- Reviewer: Arigami T, Kadayifci A **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5567-5578

DOI: 10.3748/wjg.v23.i30.5567

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

ORIGINAL ARTICLE

Retrospective Study

Serous pancreatic neoplasia, data and review

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Author contributions: Dietrich CF is accepting full responsibility for the conduct of the study, he and all others had access to the data, all had control of the decision to publish; all authos planning the study, conducting the study, collecting and/or interpreting data, drafting the manuscript, and critical revision of the manuscript for important intellectual content Statistical analysis; each author made a statement that they approved the final draft submitted.

Conflict-of-interest statement: No potential conflicts of interest relevant to this article were reported.

Data sharing statement: Each author gives permission to the graphic designers to alter the visual aspect of figures, tables, or graphs.

Manuscript source: Invited manuscript

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Received: March 5, 2017
Peer-review started: March 7, 2017
First decision: April 11, 2017
Revised: June 8, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: August 14, 2017

Abstract

AIM

To describe the imaging features of serous neoplasms of the pancreas using ultrasound, endoscopic ultrasound, computed tomography and magnetic resonance imaging.

METHODS
This multicenter international collaboration enhances a literature review to date, reporting features of 287 histologically confirmed cases of serous pancreatic cystic neoplasms (SPNs).

RESULTS
Female predominance is seen with most SPNs presenting asymptomatically in the 5th through 7th decade. Mean lesion size was 38.7 mm, 98% were single, 44.2% cystic, 46% mixed cystic and solid, and 94% hypoechoic on B-mode ultrasound. Vascular patterns and contrast-enhancement profiles are described as hypervascular and hyperenhancing.

CONCLUSION
The described ultrasound features can aid differentiation of SPN from other neoplastic lesions under most circumstances.

Key words: Guideline; Cancer; Ultrasound; Endoscopic ultrasound; Elastography

Core tip: Serous pancreatic cystic neoplasms are infrequent neoplasms of the pancreas. Ultrasound features including single cystic or mixed cystic and solid hypoechoic lesions, hypervascular and hyperenhancing profiles as described can aid differentiation from other neoplastic lesions under most circumstances.

Dietrich CF, Dong Y, Jenssen C, Ciaravino V, Hocke M, Wang WP, Burmester E, Möller K, Atkinson NSS, Capelli P, D'Onofrio M. Serous pancreatic neoplasia, data and review. *World J Gastroenterol* 2017; 23(30): 5567-5578 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5567.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5567

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas, accounting for about 90% of malignant pancreatic neoplasms. The most important imaging diagnosis to differentiate from PDAC are neuroendocrine tumours[1,2]. Most pancreatic cystic neoplasms are mucin producing including intraductal pancreatic mucinous neoplasia (IPMN) and mucinous cystic neoplasia (MCN)[3]. Other important pancreatic lesions to differentiate include metastases (*e.g.,* of renal cell cancer), lymphoma and ectopic spleen. Comparatively less is known about serous pancreatic neoplasia (SPN) which is a rare (less than 1%-2% of pancreatic neoplasia) and predominantly cystic appearing tumor of the pancreas. Critically SPN is considered benign in comparison to the majority of other common cystic tumors and all solid tumors of the pancreas. SPN has been previously termed serous pancreatic cystic neoplasia and serous pancreatic cystadenoma[4]. Approximately 75% of SPNs are found in women at an average age of 50-60 years, however SPN may also be found in much younger patients[5]. Typically located in the body-tail of the pancreas and solitary, the majority are detected incidentally[4,6-8]. In the largest series published to date (n = 2622), 61% of patients were asymptomatic and 27% reported non-specific abdominal complaints, whilst only 9% presented with pancreatobiliary symptoms, and 9% with other symptoms[5].

Histopathologically, SPNs are cyst-forming epithelial neoplasms composed of cuboidal, glycogen-rich, epithelial cells, without cellular atypia. The cyst content is defined as "clear watery". SPNs lack the genetic alterations typical of PDAC, pancreatic neuroendocrine tumors, and mucinous cystic neoplasms of the pancreas (MCN and IPMN). Rather, they are characterized by molecular alterations of the von-Hippel-Lindau (*VHL*) gene and overexpression of vascular endothelial factor (VEGF), glucose transporter 1, and other markers of clear-cell tumorogenesis (HIF1-α, CAIX)[9]. VHL patients have a high prevalence of pancreatic lesions (unclassified benign cysts, neuroendocrine tumors, SPNs) and often multiple tumors in the gland. A systematic review found SPN in 11% of VHL patients[10]. However, the majority of SPNs are sporadic.

Most serous neoplasms are benign and defined as serous cystadenomas (SCAs). More aggressive subtypes occur, demonstrated in a series of 257 resected SCAs; 5.1% of SPNs were locally aggressive, with invasion of surrounding structures or vasculature or direct extension into peripancreatic lymph nodes, while 0.8% were frankly malignant, given the presence of metastases[5]. Another series of 193 SPNs reported infiltration of adjacent organs and structures in 3% of cases, but called into question the term "malignancy" for cases of very large SPNs[11]. Rare cases of synchronous or metachronous hepatic SPNs may represent multifocal occurrence rather than metastatic spread[9]. Frankly malignant behavior with metastases seems to be very rare. In a multinational study of 2622 patients with SPNs, only 3 serous cystadenocarcinomas (SCACs) were recorded (0.1%)[8]. Therefore, SCAC is represented predominantly by a few sporadic case reports in the recent literature. Follow-up studies have not demonstrated proof of an adenoma-carcinoma sequence in SPN[8,9,11-15].

The growth rate of SPNs is variably reported. In the largest series (n = 2622) it was found to be only 4 mm/year, and size was stable or decreased in as many as 63% of patients[8]. This was supported by another series (n = 214), where the doubling time was estimated at approximately 12 years, and was independent of tumour size[14]. In contrast, in another case series (n = 106) growth rate was reported to vary by tumor

size, with the fastest growth (20 mm/year) observed in tumors ⩾ 40 mm, compared to tumors < 40 mm (increasing 12 mm/year)[16]. Finally, a fourth series (n = 145) found the fastest growth 7-10 years after diagnosis (60 mm/year) compared to the first 7 years (10 mm/year). Oligocystic or macrocystic appearance, a history of other tumors, and patient age were all significant predictors of more rapid tumor growth[13].

Diagnosis of SPN primarily is based on imaging [computed tomography (CT); magnetic resonance imaging (MRI); ultrasound (US); endoscopic ultrasound (EUS)]. The classical microcystic SPN consists of innumerable very small cysts separated by thin, vessel-containing fibrous septae. Cysts may be microscopic or measure up to 10 mm. These features cause a honeycomb or sponge-like appearance with hypervascularity and distinct, sometimes lobulated, margins. Pertinent negatives include communication with the pancreatic duct, vascularized mural nodules, and a hypervascular capsule on contrast-enhanced imaging, whilst a central scar is visible in a third of cases and may contain calcifications[17-25]. Pitfalls may arise from several factors: the macro- and oligo-cystic types of SPN can appear similar to pseudocysts or MCN. Rarely the solid form of SPN may be confused with other hypervascular well-circumscribed pancreatic tumors, in particular neuroendocrine tumors and solid pseudopapillary neoplasms[18,19,26-28]. In contrast to the mentioned reports, an atypical appearance on CT was found in 61.1% of cases in a study of 72 confirmed SPNs[28].

A correct diagnosis of SPN is challenging. The preoperative diagnosis was wrong in 63% of resected cases in a Japanese series[12], and in a large multinational study the indication for surgery was an uncertainty of diagnosis in 60% of cases[8]. To date, no large series describing typical and atypical US- and EUS-features of SPNs has been reported.

The aim of this retrospective study was to describe the imaging features of serous neoplasms of the pancreas using US, EUS, CT and MRI. The frequency of atypical imaging aspects by different imaging modalities will be estimated and the most common atypical features will be reported, particularly of US which is often the initial imaging modality employed.

MATERIALS AND METHODS

Patients

An international multicenter retrospective data collection of 287 histologically confirmed cases of SPNs was performed. The cohort was not uniform according to the resection criteria. No other exclusion criteria have been defined.

Examination technique

Conventional ultrasound and contrast enhanced ultrasound (CEUS) were performed in all patients with one of six ultrasound systems: Philips iU22 unit (Philips Bothell, WA, United States; C5-1 convex array probes, 1-5 MHz), or LOGIQ E9 (GE Healthcare, Milwaukee, WI, United States; C1-5 convex array probes, 1-5 MHz) or Hitachi (Hi vision EUB-6500, Preirus, Ascendus; C715 convex array probes, 1-5 MHz), or SIEMENS (Acuson Sequoia or S2000), or Toshiba (Aplio platinum 500; Aplio CV, convex array probes 3-6 MHz). CEUS was performed using contrast harmonic real-time imaging at a low MI 0.05-0.30. The ultrasound contrast agent Sonovue was used at a dose of 1.5-2.4 mL, immediately followed by an injection of 10 mL sodium chloride solution. Images were recorded for 3 min after contrast agent injection.

Contrast enhanced EUS was performed using longitudinal echoendoscopes EG-3870 UTK and Hitachi platforms (Hitachi HI vision EUB-6500, Hitachi Preirus, Hitachi Ascendus)[29-32].

Imaging Evaluation (TUS, EUS, CEUS, ceEUS)

After identification of the pancreatic lesion by conventional B-mode US or EUS, contrast enhanced imaging was immediately performed. All examinations were interpreted according to the 2011 EFSUMB guidelines[1]. CEUS features of pancreatic lesions were compared to the surrounding normal pancreatic parenchyma.

Final diagnoses, treatment and clinical follow up

Most patients (n = 249, 86.7%) were diagnosed as SPNs by post-operative histopathology. 31 (10.8%) cases were confirmed by EUS FNA and 7 (2.5%) by transabdominal (percutanous) ultrasound-guided core needle biopsy (18-gauge 20-cm single-use biopsy needles; Temno, Germany, or BioPince, Pflugbeil, Germany). Clinical follow-up for a minimum of 12 mo was established for all patients with SPN diagnosed by biopsy. Additional information on outcome data was not requested.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 17.0 (SPSS Inc., Chicago, IL, United States). The χ^2 test and Fisher's exact test were used to compare categorical parameters between the groups. Continuous parameters were presented as the mean ± SD, and Student's t test was used. A P value of less than 0.05 was considered statistically significant.

RESULTS

Epidemiology

The average age of included patients was 57.3 ± 14.2 years (18-85 years). Fifty-eight patients were male and 229 were female (Table 1).

Conventional ultrasound

On conventional B mode ultrasound (BMUS) most SPN lesions (n = 113, 39.3%) were detected in the head/neck of the pancreas. Most SPN lesions (97.9%) were

Table 1 Baseline characteristics of serous pancreatic neoplasia patients

Characteristic	SPN patients (n = 287)
Age (yr)	
mean ± SD	57.3 ± 14.2
Range	18-85
Female/Male	229/58
Symptoms	
Pancreatitis	5
Weight loss	9
Anemia	1
Incidental finding	272
Histological results	
Surgery	249
EUS FNA (22G)	31
TUS-Bx (18 G)	7

SPN: Serous pancreatic neoplasia; EUS: Endoscopic ultrasound; FNA: Fine needle aspiration; TUS-Bx: Transabdominal biopsy.

Table 2 Conventional B mode ultrasound findings of serous pancreatic neoplasia n (%)

Characteristic	SPN lesions (n = 287)
Location	
Head/neck	113 (39.3)
Body	89 (31.0)
Tail	85 (29.6)
Size of lesions (mm)	
mean ± SD	38.7 ± 26.2
Range	4-160
Number of lesions	
Single	281 (97.9)
Multiple	6 (2.1)
B mode aspect	
Microcystic mix	31 (10.8)
Macrocystic	96 (33.4)
Solid and cystic	133 (46.3)
Solid	27 (9.4)
B mode echogenicity	
Anechoic	10 (3.4)
Hypoechoic	271 (94.4)
Hyperechoic	6 (2.2)
CDI vessel detectable	
Avascular	228 (79.4)
Macrovessels detectable	59 (20.6)
CDI vascular pattern (n = 59 with macrovessels)	
Central artery	26 (44.1)
Typical spoke wheel appearance	21 (35.6)
No specifics	12 (20.3)

SPN: Serous pancreatic neoplasia; CDI: Color Doppler imaging.

single, though 6 (2.1%) patients had multiple lesions, and most lesions (97.9%) were hypoechoic on BMUS. With colour Doppler imaging (CDI), macrovessels were detected in 20.6% of lesions, among which the typical "spoke wheel" appearance was identified in 35.6% of lesions (Table 2).

CEUS

Transabdominal CEUS was performed in 173 (60.3%) lesions. After contrast agent injection, most SPN lesions displayed hyper- (36.5%) or isoenhancement (61.8%) in the arterial phase. During the late phase, most SPN lesions were hyper-enchancing (22.5%) or iso-enhancing (74.9%) (Table 3).

Table 3 Contrast enhanced ultrasound imaging features of serous pancreatic neoplasia lesions n (%)

Characteristic	SPN lesions (n = 173)
Arterial phase	
Hyperenhancement	63 (36.5)
Isoenhancement	107 (61.8)
Hypoenhancement	3 (1.7)
Late phase	
Hyperenhancement	39 (22.5)
Isoenhancement	129 (74.6)
Hypoenhancement	5 (2.9)

CEUS: Contrast enhanced ultrasound; SPN: Serous pancreatic neoplasia.

EUS and contrast enhanced EUS

EUS was performed in 61 patients diagnosed with SPN. Using CDI, macrovessels were detected in all 61 cases. Contrast enhanced endoscopic ultrasound (CE-EUS) was performed in 54 SPN lesions, demonstrating hyper-enhancement in all cases (Table 4).

DISCUSSION

SPNs are less frequent than common pancreatic tumors such as solid ductal adenocarcinoma and cystic IPMN but recent estimates suggest SCAs represent about 20% of all cystic pancreatic lesions[33,34]. SPN typically present as a solitary multilocular micro-cystic lesion with a honeycomb architecture due to the presence of multiple microcysts. Thin walls and multiple thin septa orient toward the centre/scar of the lesion, without communication with the main pancreatic duct. In typical cases an imaging diagnosis can be made confidently. However, atypical presentations are commonly encountered in everyday clinical practice. Specifically, extremely microcystic SPNs are considered rare, resembling a solid lesion in conventional US, but in fact a solid component was seen in the 55.7% of cases in our multicentre study. After contrast administration, these solid SPNs may resemble hypervascular solid lesions with homogeneous hyperenhancement, making differentiation from neuroendocrine neoplasms as difficult as it is crucial[35,36]. MRI may reveal a lesion's true cystic nature[37]. The macrocystic variant must be differentiated from other macrocystic pancreatic lesions, such as pseudocyst, mucinous cystic neoplasms, side-branch and mixed type IPMNs, solid tumors (either adenocarcinomas or neuroendocrine) with cystic degeneration, and lymphangiomas[38-40].

Epidemiology

The mostly asymptomatic SPN is often a solitary lesion with multilocular cysts predominantly in the corpus and tail of the pancreas. SPNs are usually diagnosed

Table 4 Endoscopic ultrasound and contrast enhanced endoscopic ultrasound imaging features of serous pancreatic neoplasia lesions *n* (%)

Characteristic	SPN lesions (n = 61)
EUS	
Anechoic	2 (3.3)
Hypoechoic	56 (91.8)
Isoechoic	3 (4.9)
EUS-CDI vessel detectable	
Avascular	0
Macrovessels detectable	61 (100)
EUS-CDI vascular pattern (n = 61)	
Central artery	26 (42.6)
Typical spoke wheel appearance	15 (24.6)
No specifics	20 (32.8)
CE-EUS (n = 54)	
Hyperenhancement	54 (100)
Isoenhancement	0
Hypoenhancement	0
Final EUS-Diagnosis	
"Eyecatcher"	49 (80.3)
Typical SCA	5 (8.2)
Unclear macrocyst	6 (9.8)

EUS: Endoscopic ultrasound; EUS-CDI: Endoscopic ultrasound color Doppler Imaging; CE-EUS: Contrast enhanced endoscopic ultrasound; SPN: Serous pancreatic neoplasia; SCA: Serous cystadenoma.

in females in the 5th to 7th decade (female to male ratio 2-3:1) but with improved imaging methods the neoplasia may be diagnosed much earlier[3]. Multiple lesions, including involvement of the entire organ, have been observed in patients with Von Hippel-Lindau disease[6,7,10,23]. SPNs may present up to 20% of cystic pancreatic lesions[3,33,41].

Clinical symptoms

Sporadic and benign SPNs are most often an incidental finding without symptoms; jaundice is particularly uncommon. In our series only 5.2% of SPNs presented with symptoms. During the course of the disease, symptoms may be caused by growth of the lesion. The main pancreatic duct and or common bile duct may become entrapped in the lesion, especially if large in dimension. The reported growth rate has been estimated at 4 mm per year[39].

Pathology

The solitary well demarcated and multicystic SPN is a multilobular cyst forming epithelial neoplasia with a somewhat "honeycomb" architecture (Figure 1) without communication with the pancreatic duct.

Histologically, monostratified cuboidal, glycogen rich epithelial cells typically without mitoses are observed. Centrally located fibrous tissue (a so called "scar") with or without calcifications can be found, similar to focal nodular hyperplasia of the liver; therefore the lesion has been referred to as "FNH of the pancreas"[4]. SPNs are typically hypervascular lesions, where septa are characterized by abundant subepithelial micro- and macro-vessels[6,7].

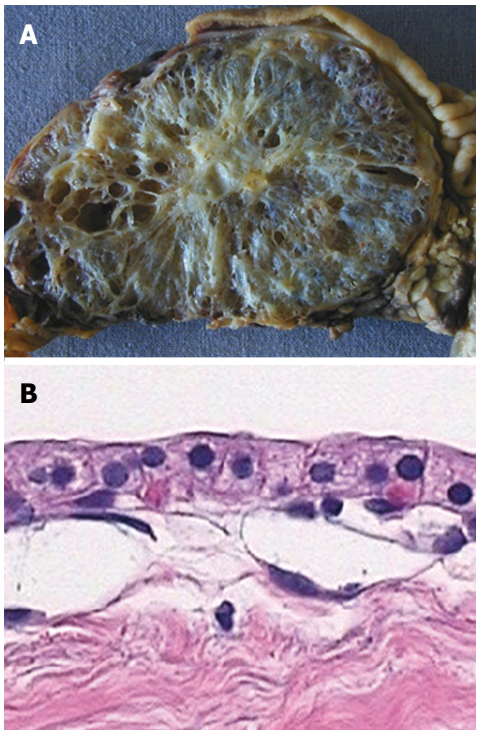

Figure 1 Macro- and micro-pathology (histology, cytology) of microcystic pancreatic adenoma. A: Typical microcystic appearance of serous cystadenoma with "honeycomb" architecture, and central scar with small calcification; B: Histology demonstrates the typical single layer of clear cuboidal epithelial cells lining the cysts.

Size of single cysts

According to the size of the cysts, SPNs can be classified as real solid lesions (< 5%), pseudo-solid SPNs (cysts only detectable by microscopic evaluation), microcystic (< 10 mm), oligocystic (< 20 mm), and macrocystic appearance (30%)[4,6,7,42,43]. The cystic appearance can be described by thin multiple septa oriented toward the center of the lesion. Mixed forms (microcystic and macrocystic) are typical in large SPNs. Macrocystic giant SPNs are more commonly located in the pancreatic head, and a male preponderance was observed. The macrocystic variant may be indistinguishable from other macrocystic tumors of the pancreas[44].

Malignant transformation

SPN is typically a benign neoplasia but in a series of 257 resected SPNs, local expansion (5.1%) and malignant transformation with metastases (0.8%) have been described[5]. Therefore, follow-up is recommended by means of ultrasound or MRI. Surgical treatment is recommended only for symptomatic patients or patients with growing lesions, usually larger than 4 cm[16].

Imaging

Ultrasound: Sonographically, SPN is a typically lobular cyst forming isoechoic neoplasia with centrally oriented thin walls (thin septae) without communication with the main pancreatic duct. A central hypoechoic spot (central fibrovascular scar) is characteristic[3,45-47]. In

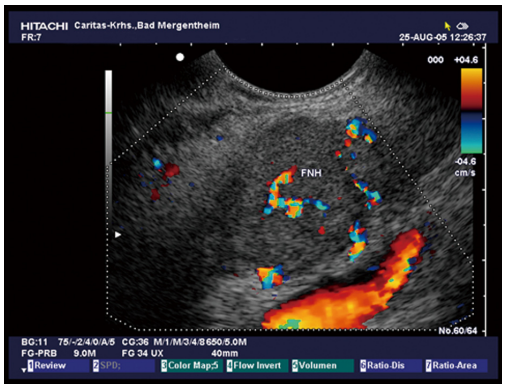

Figure 2 Typical microcystic serous pancreatic neoplasia using colour Doppler imaging. Note the centrally located artery.

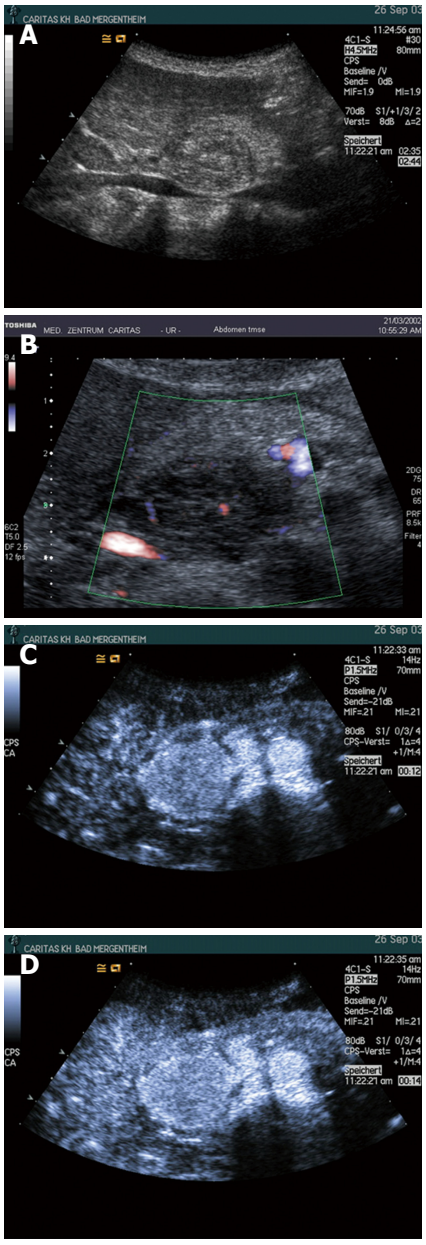

Figure 3 Typical microcystic serous pancreatic neoplasia using B-mode (A), colour Doppler imaging (B), and contrast enhanced ultrasound (C and D). Note the centrally located artery and the typical hyperenhancement.

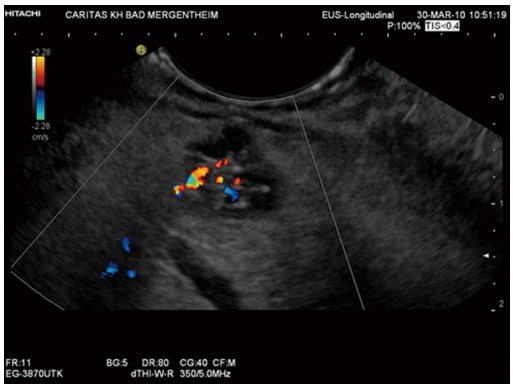

Figure 4 Typical oligocystic serous pancreatic neoplasia using endoscopic ultrasound.

the case of depictable cysts the content is anechoic. SPNs are typically hypervascular lesions since the septa are composed by abundant subepithelial micro- and macro-vessels[6,7] (Figures 2 and 3).

As has been shown in a prospective study (n = 12) using CE-EUS, hypervascularity, sharp delineation, fibrotic strands and typical vessel architecture are the predominant features of serous microcystic adenoma[47] (Figures 4 and 5).

Solid SPNs may mimic neuroendocrine tumours, renal metastases, intrapancreatic accessory spleens and other hypervascular pancreatic tumours[35,36,45,48]. Solid and pseudosolid SPNs are typically hypervascular and, therefore, hyperenhancing using CEUS[45,48,49,50].

EUS: EUS is an accurate imaging modality to diagnose and exclude neoplasia of the pancreas[1,51-53]. The features are the same as described for conventional ultrasound[54]. SPN do not communicate with the main pancreatic duct but may show proximal duct dilatation due to compression, whereas IPMN usually showed distal or whole pancreatic duct dilatation[50,55]. CE-EUS has been proven to be of value for many indications[29-32,56-60]. In our study EUS was able to detect macrovessels and hypervascularity by CDI contrast-enhanced imaging in 100% of cases, whereas percutaneous US with CDI delineated macrovessels only in 20.6% of cases.

Endoscopic ultrasound fine needle aspiration: Endoscopic ultrasound fine needle aspiration (EUS-FNA) of SPN should target the largest cyst for fluid analysis[61,62]. The cyst fluid is watery (non-viscous) and colorless[63]. The cellularity is low with few cuboidal glycogen positive and mucin negative epithelial cells. CEA levels are usually but not always low (< 20 ng/mL). In the majority of cases, aspirated fluid will be hypocellular with few groups of bland cuboidal epithelial cells embedded in granular debris[64-67]. Round to cuboid serous epithelial cells with clear cytoplasma and small, round nuclei forming loose clusters or monolayered sheets are identified in only 20%-25% of cases[64,66,68]. Positive α-inhibin immunocytochemistry

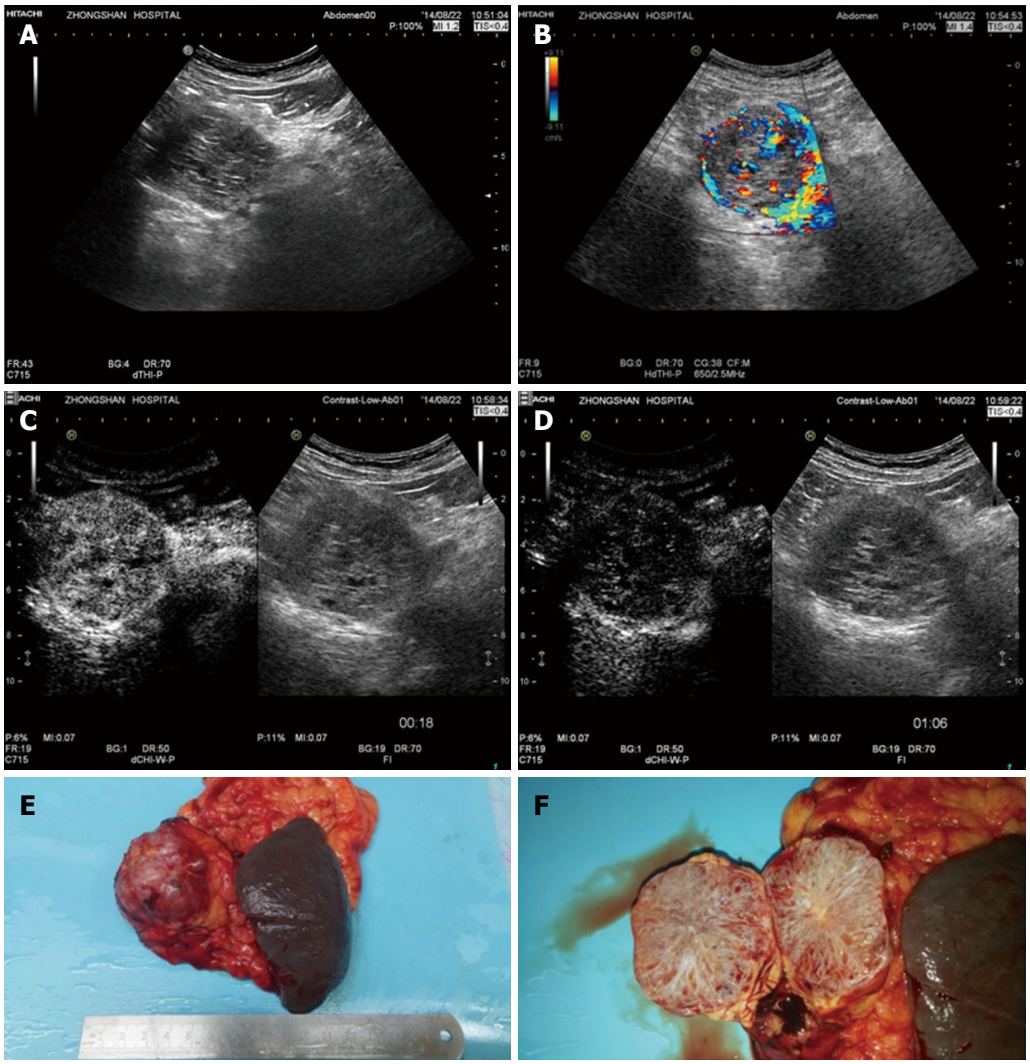

Figure 5 Histopathologically proven serous microcystic serous pancreatic neoplasia. A: A solid-cystic lesion was detected in the head of pancreas with B-mode ultrasound; B: Multiple interlesional color flow signals were detected using colour Doppler imaging; C: Contrast enhanced ultrasound showed the lesion to hyperenhance in the arterial phase; D: Isoenhance in the late phase; E and F: Surgical pathology shows the typical honeycomb structure.

may enhance the diagnostic accuracy of EUS-FNA in SPN[66]. Promising cyst fluid markers with high sensitivity and specificity for SPN include VEGF-A and a molecular assay for KRAS, GNAS and VHL mutations. In one study, VEGF-A was markedly elevated in SPN when compared to pseudocysts and mucinous neoplastic cysts[69,70]. The presence of KRAS and GNAS mutations is highly specific for IPMN and is never observed in SPN, whereas VHL deletions are found in almost all SPN[69,71-74].

Core biopsy: In solid and pseudosolid lesion we prefer histological evaluation which allows definite diagnosis[51,52,75].

MRI: The MRI features of SPNs are also represented by a typical lobular "honeycomb" shaped contour and architecture with thin walls less than 2 mm, in contrast to other cystic neoplasia of the pancreas. SPNs are homogeneously hypointense on T1-weighted MRI sequences. The cystic nature of the lesion can be easly demonstrated by a typical hyperintense signal on T2-weighted images. The hyperintense cysts are surrounded by hypointense septa and sometimes by a hypointense (pathognomonic) central scar. The central scar is a less sensitive (15%) but specific sign of SPN[39,45,76-79].

In contrast to EUS, the individual vessels cannot be displayed but contrast enhanced MRI using gadolinium chelates also reveal the hypervascular nature by diffuse hyperenhancement in pseudosolid SPN However also in pseudosolid SPN, MRI remains highly accurate in showing the cystic nature of the lesion on T2-weighted images[50]. The macrocystic types present features similar to other macrocystic tumors of the pancreas, but the lobulated contours, together with the absence of wall enhancement and wall thickness less than 2 mm, should suggest the correct diagnosis[39,46,76,79].

CT: CT is sometimes helpful for detection of SPN but

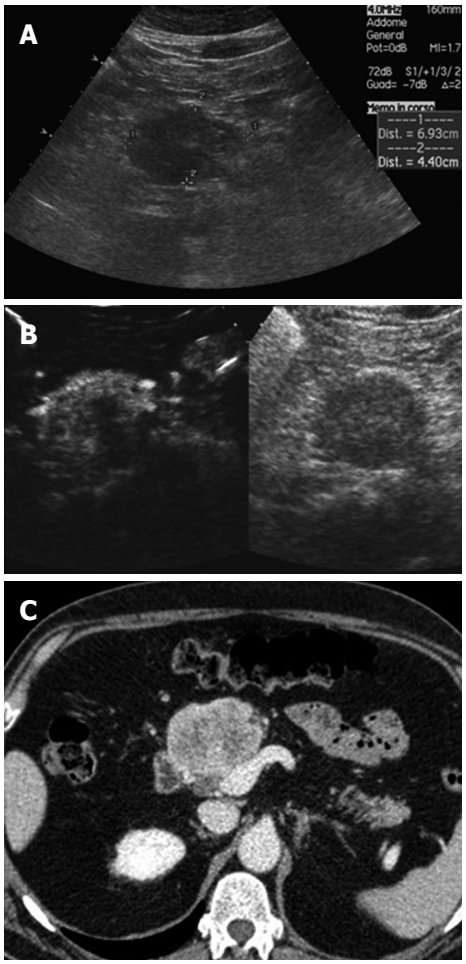

Figure 6 Pseudo-solid serous pancreatic neoplasia, histologically demonstrated to have a microcystic structure. A: B-mode ultrasound shows a solid hypoecoic mass in the neck of the pancreas; B: Contrast enhanced ultrasound shows the lesion to hyperenhance with a hypoechoic defect in the center; C: Computed tomography shows the lesion as solid and inhomogeneously hyperenhancing.

should in general not be used for the evaluation and differential diagnosis of cystic pancreatic lesions. CT might be helpful in the visualization of a centrally located calcified scar. SPN may mimic a hypervascular lesion[17,39,46].

Differential diagnosis

The presence of a unilocular lobulated cyst located in the pancreatic head with anechoic fluid and wall thickness less than 2 mm are indicative of SPN using all imaging methods and should be considered as a unilocular macrocystic SCA, until otherwise proven (Figures 6-8)[80].

Pancreatitis: A clinical history of pancreatitis is crucial for differentiating pseudocysts. Imaging findings of pancreatitis derived pseudocysts include signs of inflammation in the acute setting, calcifications, thin-walled duct dilation, pancreaticolithiasis, and atrophy and typical dilation of the pancreatic duct[39,51,81,82].

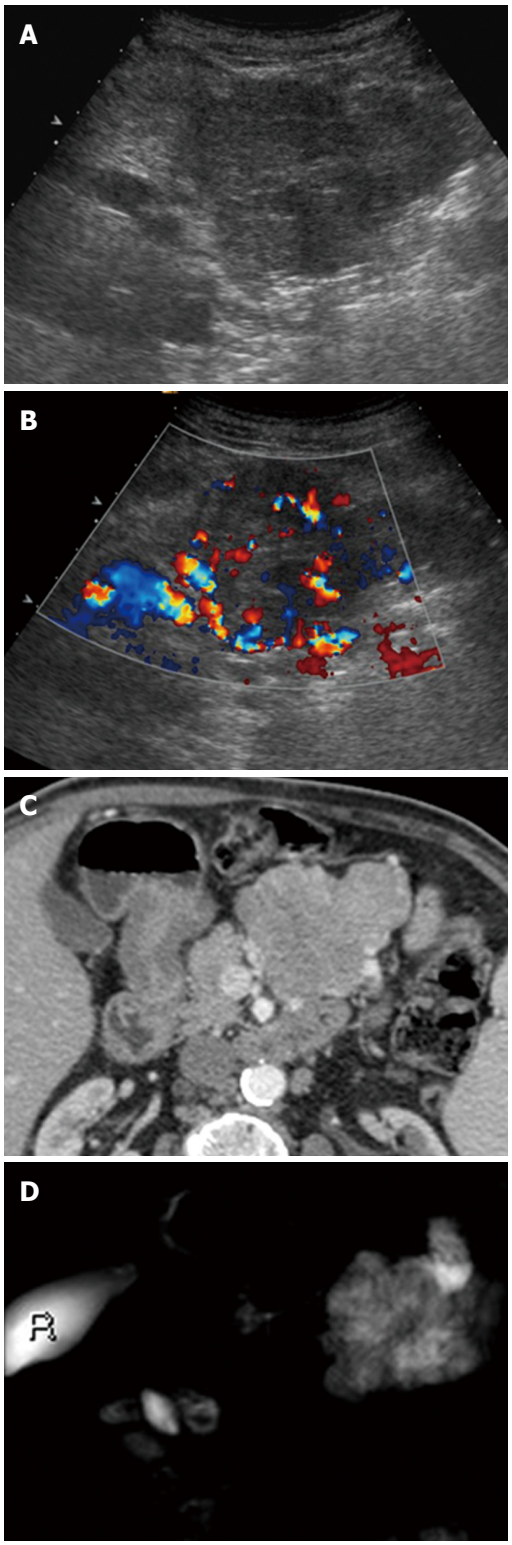

Figure 7 Large pseudosolid serous pancreatic neoplasia. A: With B-mode ultrasound a huge mass is visible appearing solid and inhomogeneously hypoechoic; B: Doppler shows large arterial vessels within the mass; C: With Computed tomography the lesion appears pseudosolid with inhomogeneous slight enhancement; D: Magnetic resonance imaging clearly shows the cystic nature of the mass with microcystic appearance.

Solid SPNs are often misdiagnosed as chronic (focal) pancreatitis, which is important to know.

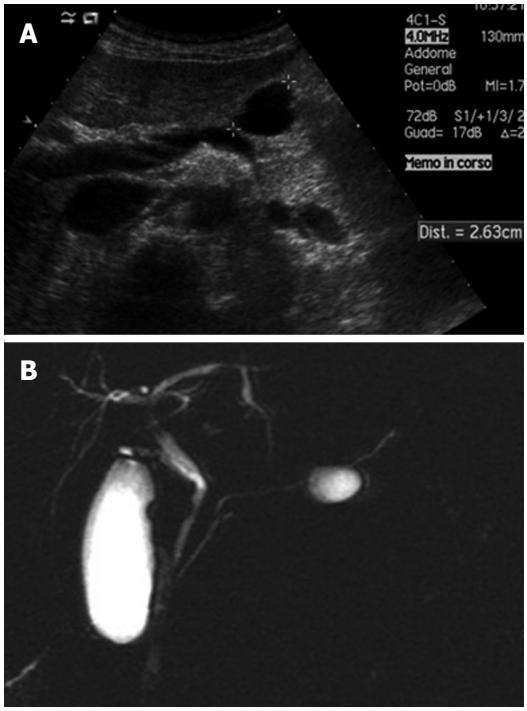

Figure 8 Unilocular serous pancreatic neoplasia. A: B-mode ultrasound shows a cyst in the body of the pancreas; B: Magnetic resonance imaging shows small cystic lesions in the body of the pancreas not communicating with the main pancreatic duct.

IPMN: Side branch or mixed type IPMN typically communicate with the pancreatic duct system and therefore can be differentiated from non-communicating SPN and MCN.

MCN: MCNs are also most common in females and they do not communicate with the pancreatic duct, but are usually located in the pancreatic tail[83]. The complex internal architecture of MCNs including septa and mural nodules can be best visualized using EUS and contrast enhanced EUS, but cMRI may be also helpful. In EUS-FNA, mucin and evaluation of the CEA level is important[39]. Peripherally located eggshell calcifications are a specific sign of MCN[84]. Other rare differential diagnoses include solid papillary neoplasia and lymphoepithelial cysts[39].

In conclusion, serous pancreatic neoplasms are an important differential of cystic, mixed and solid pancreatic lesions, with a generally benign course. This large series of histologically proven cases demonstrates the typical demographic, structural, vascular and contrast enhancement features which can distinguish these lesions from more common pathologies.

COMMENTS

Background

Serous pancreatic cystic neoplasms are infrequent neoplasms of the pancreas. A correct diagnosis of pancreatic cystic neoplasms (SPNs) is challenging. To date, no large series describing typical and atypical ultrasound (US)- and endoscopic ultrasound (EUS) -features of SPNs have been reported.

Research frontiers

The aim of this retrospective study was to describe typical and atypical imaging features of serous neoplasms of the pancreas using US, EUS, computed tomography and MRI.

Innovations and breakthroughs

This multicenter international collaboration reports on imaging features in one of the largest series of 287 histologically confirmed cases of SPNs.

Applications

Ultrasound B-mode descriptors and contrast enhanced ultrasound describing the vascular pattern and enhancement features are helpful to differentiate SPN from other neoplastic cystic lesions.

Peer-review

Very interesting study about the serous pancreatic neoplasia.

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P- Reviewer: Lowenberg M, Reich K **S- Editor**: Qi Y **L- Editor**: A
E- Editor: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5579-5588

DOI: 10.3748/wjg.v23.i30.5579

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

ORIGINAL ARTICLE

Retrospective Study

Pancreaticoduodenectomy for duodenal papilla carcinoma: A single-centre 9-year retrospective study of 112 patients with long-term follow-up

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Author contributions: Lian PL designed the research, analyzed the data, drafted and revised the paper; Wang XQ, Xu XC and Zhao Z performed the research; Chang Y searched the literature; Xu KS revised the paper, approved the final version.

Institutional review board statement: The study was reviewed and approved by the PLA General Hospital Institutional Review Board.

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

Conflict-of-interest statement: We declare that there are no conflicts of interest to disclose.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: May 3, 2017
Peer-review started: May 4, 2017
First decision: June 1, 2017
Revised: June 13, 2017
Accepted: June 18, 2017
Article in press: June 19, 2017
Published online: August 14, 2017

Abstract

AIM

To retrospectively evaluate the factors that influence long-term outcomes of duodenal papilla carcinoma (DPC) after standard pancreaticoduodenectomy (SPD).

METHODS

This is a single-centre, retrospective study including 112 DPC patients who had a SPD between 2006 and 2015. Associations between serum levels of CA19-9 and CEA and various clinical characteristics of 112 patients with DPC were evaluated by the χ^2 test and Fisher's exact test. The patients were followed-up every 3 mo in the first two years and at least every 6 mo afterwards, with a median follow-up of 60 mo (ranging from 4 mo to 168 mo). Survival analysis was conducted using the Kaplan-Meier survival and Cox proportional hazards model analysis. The difference in survival curves was evaluated with a log-rank test.

RESULTS

In 112 patients undergoing SPD, serum levels of CA19-9 was associated with serum levels of CEA and drainage mode (the *P* values were 0.000 and 0.033, respectively); While serum levels of CEA was associated with serum levels of CA19-9 and differentiation of the tumour (the *P* values were 0.000 and 0.033, respectively). The serum levels of CA19-9 and CEA were closely correlated (χ^2 = 13.277, *r* = 0.344, *P* = 0.000). The overall 5-year survival was 50.00% for 112 patients undergoing SPD. The Kaplan-Meier survival analysis showed that increased serum levels of CA19-9, CEA, and total bilirubin were correlated with a poor prognosis, as well as a senior grade of infiltration depth, lymph node metastases, and TNM stage(the *P* values were 0.033, 0.018, 0.015, 0.000, 0.000 and 0.000, respectively). Only the senior grade of infiltration depth and TNM stage retained their significance when adjustments were made for other known prognostic factors in Cox multivariate analysis (*RR* = 2.211, *P* = 0.022 and *RR* = 2.109, *P* = 0.047).

CONCLUSION

For patients with DPC, the serum levels of CA19-9 and CEA were closely correlated, and play an important role in poor survival. Increased serum levels of total bilirubin and lymph node metastases were also correlated with a poor prognosis. The senior grade of infiltration depth and TNM stage can serve as independent prognosis indexes in the evaluation of patients with DPC after SPD.

Key words: Duodenal papilla carcinoma; CA19-9; CEA; Survival; Pancreaticoduodenectomy

Core tip: For duodenal papilla carcinoma (DPC), standard pancreaticoduodenectomy (SPD) is still the most important treatment. However, the prognosis assessment for DPC after SPD is not yet clear. So we conducted a long-term follow-up and observation with a large sample. Our study demonstrated that the serum levels of CA19-9 and CEA were closely correlated and played an important role in poor survival. Increased serum levels of total bilirubin and lymph node metastases were also correlated with a poor prognosis. The senior grade of infiltration depth and TNM stage can serve as independent prognosis indexes for patients with DPC after SPD.

Lian PL, Chang Y, Xu XC, Zhao Z, Wang XQ, Xu KS. Pancreaticoduodenectomy for duodenal papilla carcinoma: A single-centre 9-year retrospective study of 112 patients with long-term follow-up. *World J Gastroenterol* 2017; 23(30): 5579-5588 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5579.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5579

INTRODUCTION

The incidence rate of primary duodenal papilla carcinoma (DPC) is low, only accounting for 0.01% of malignant tumours and accounting for 5% of gastrointestinal malignant tumours[1]. It has been reported in the literature that among malignant tumours primarily occurring in the duodenum, 60% are diagnosed as DPC[2], and the incidence rate of DPC in periampullary carcinoma is the highest. A series of studies have demonstrated that there is a higher excision rate and better prognosis of DPC than other malignant tumours around the duodenal ampulla, and the survival rate within 5 years after the operation is in the range of 50%-60%[3].

For DPC, Standard pancreaticoduodenectomy (SPD) is still the most important treatment mode[4-6]. Based on a large number of studies, there are still many disputes concerning the prognosis assessment for primary DPC after SPD, and there is lack of long-term follow-up and observation of a large sample.

Therefore, the main objective of this study was to review and report our own single-centre data of 112 patients with DPC at the PLA General Hospital between 2006 and 2015 to evaluate factors influencing outcome after radical SPD surgery.

MATERIALS AND METHODS

General data

A total of 112 patients with DPC who received SPD in the PLA General Hospital from August 2006 to November 2015 were enrolled. In this study, all patients were confirmed as DPC according to postoperative pathological examinations. There were 74 males and 38 females, with a median age of 57.95 years. The disease course was 0.13-15 years.

This study only enrolled patients who received SPD due to DPC. The following patients were not enrolled: patients who had received radiotherapy and chemotherapy before the operation; patients who received endoscopic local excision of benign tumours of the duodenum; patients who could not tolerate SPD because of body conditions; and patients with complicated malignant tumours at other sites.

All patients and/or a family member signed a written informed consent form, in which the nature of the diseases, possible therapeutic methods and postoperative potential complications were detailed. This study was approved by the ethics committee of the PLA General Hospital and was performed in accordance with the ethical standards specified in the 1964 Declaration of Helsinki and its later amendments.

Clinical manifestations and concomitant diseases

The most common clinical manifestation was jaundice; other symptoms included body weight decreases and epigastric discomfort. In addition, there were

20 patients with cholangitis symptoms such as intermittent or acute fever. Common concomitant diseases included hypertension, heart diseases and diabetes mellitus. The medical history of other patients included 5 cases of endoscopic local excision and4 cases of choledocholithotomy.

Preoperative evaluation

Routine blood tests were carried out for all patients before the operation, including blood routine, hepatic and renal function, biochemical indicators, blood coagulation series and tumour markers.

There were 87 patients with increased bilirubin more than 17.1 μmol/L, of whom there were 69 patients with increased bilirubin more than 34.2 μmol/L. Preoperative tests of tumour markers mainly included CA19-9 and CEA. There were 39 patients with CA19-9 higher than 120 U/mL, while there were only 16 patients with CEA higher than 5 ng/mL.

Preoperative routine ultrasound Band CT examinations were carried out. Intrahepatic and extrahepatic bile duct extension suggested that low-level biliary obstruction was the most common manifestation on CT. Preoperative endoscopic retrograde cholangiopancreatography (ERCP) examinations were carried out for 92 patients, during which the conditions of duodenal papilla were directly observed under an endoscope, and in 64 of them, biopsy and pathological examinations were performed before the operation, which confirmed the pathological diagnosis. In 34 patients, a biliary tract prosthesis or a drainage tube was inserted during ERCP to drain bile as an active preoperative preparation measure.

Operation procedures

SPD was carried out for all patients. During the operation, it was found that in 2 cases, there was remote lymph node metastases, which made radical SPD impossible; therefore, they were excluded. For all pancreas stumps, anastomosis of the pancreatic duct and jejunum was carried out, and the anastomosis modes were categorized into anastomosis of the pancreatic duct and jejunal mucous membrane and invaginated pancreaticoenterostomy according to the diameter of the pancreatic duct and the size of pancreas amputation stump, with 90 cases and 22 cases, respectively. Intraoperative exploration found that in 34 cases, the diameter of the main pancreatic duct was greater than 4 mm; in 78 cases, it was smaller than 4 mm. For the intraoperative anastomosis of the pancreatic duct and jejunum, one end of a support tube was placed in the main pancreatic duct, and the other end was placed inside the jejunum or outside the abdominal wall to drain liquid. According to the position of the support tube, pancreatic duct drainage modes were divided into internal drainage and external drainage, with 85 cases and 27 cases, respectively. According to the different habits of operators, the jejunum input side to the output side anastomosis (Braun anastomosis) was added, with the position 8 cm under the gastro-intestinal anastomotic stoma. In 54 cases, Braun anastomosis was added. All operations were performed by chief physicians with rich experience.

Postoperative complications

For all patients, conventional postoperative treatment of the pancreas was carried out. Before being transferred back to the patients' rooms, they were observed for at least one day in the intensive care unit. After the operation, 100 μg of octreotide was subcutaneously injected three times daily in all patients for 7 continuous days. On the second day after the operation, routine blood, liver and kidney function tests were carried out; on the third day after the operation, an abdominal Colour Doppler Ultrasound examination was carried out; and 7 d after the operation, an abdominal CT examination was carried out to observe the conditions of the abdomen. Before the end of the operation, a drainage tube was placed in the pancreaticoenteric anastomosis, cholecysto-colonic anastomosis and gastrojejunostomy anastomosis, and the amount, colour and description of the draining liquid were recorded every day.

After the operation, the pancreaticoenteric drainage tube was removed when the amylase level was less than 300 U/L (less than two times the serum amylase level) inside the drainage tube, the drainage amount was less than 50 mL each day, or the drainage duration exceeded 10 d after the operation.

According to the diagnosis criteria defined by the International Study Group on Pancreatic Fistula (ISGPF)[7], a pancreatic fistula was defined as follows: 3 or more days after the operation, the draining liquid could be measured, and the activity of amylase was 3 times higher than that of the serum amylase activity. Pancreatic fistulas consisted of three grades (Grades A, B, and C) according to the clinical events of the patients' hospitalizations. Grade A pancreatic fistulas required no change from the normal clinical approach, did not delay discharge, and usually could be resolved through the removal of the retained operation drainage tube. Grade B pancreatic fistulas required a change of treatment strategy or adjustment of the clinical approach (for instance, fasting, total parenteral nutrition support, or the addition of antibiotics or somatostatin), delayed discharge, or needed readmission for treatment after discharge. If, according to the patients' pathogenetic conditions, invasive procedures were needed, the grade of the pancreatic fistula was upgraded to Grade C. Grade C pancreatic fistulas required a significant change of the treatment strategy or adjustment of the clinical approach; if clinical symptoms were aggravated and there were complications, such as sepsis and organ dysfunction, exploration through reoperation might be needed. Grade C pancreatic fistulas were often accompanied by complications, leading to an increased probability of

postoperative death.

A biliary fistula was diagnosed if there was persistent secretion of bilirubin-rich drainage fluid of more than 50 mL per day or if secretion continued after the 10th postoperative day[8].

Postoperative bleeding was defined as the need for more than 2 units of red blood cells more than 2.4 h after surgery or relaparotomy for bleeding.

The nasogastric tube was removed when the drainage decreased to less than 200 mL per 24 h[8].

Delayed gastric emptying was defined as gastric stasis requiring nasogastric intubation for 10 or more days or the inability to tolerate a regular diet on the 14th postoperative day[9].

Pathology

All excised specimens were examined in detail by two independent pathological experts; the contents observed included the nature of the tumours, size, infiltration depth, peripheral bile duct, nerves and pancreatic tissue infiltration, lymph node metastases, conditions of the tissue incisal margin (including common bile duct incisal margin, pancreas incisal margin, portal vein and mesenteric blood vessels incisal margin, stomach and jejunum incisal margin),tumour staging, *etc*. The TNM stage was done according to the UICC standard, version 7[10].

According to the measurement of postoperative gross specimens, there were 36 cases with a diameter greater than 2 cm and 76 cases with a diameter smaller than 2 cm. In 24 patients, lymph node metastases were positive after the operation, and in 88 patients lymph node metastases were negative. The range of lymph node metastases included pancreas peripheral lymph nodes (10 cases), duodenum peripheral lymph nodes (8 cases), common bile duct peripheral lymph nodes (4 cases), and superior mesenteric lymph nodes (2 cases).

According to the infiltration depth of tumours into the duodenum wall, tumours involved the superficial muscular layer (14 cases),deep muscular layer or full-thickness (34 cases).While for tumours penetrating the intestinal wall and infiltrating the peripheral tissues, the peripheral tissues that were mainly involved included pancreas (35 cases), the bile duct (24 cases), nerves (5 cases), *etc*.

Follow up

Telephone and outpatient follow-ups were performed. The patients were followed-up every 3 mo in the first two years and at least every 6 mo afterwards, with a median follow-up of 60 mo (ranging from 4 mo to 168 mo). When necessary, re-examinations by CT and MRI were carried out.

Statistical analysis

Analysis was carried out with SPSS 16.0 statistical software. Associations between serum levels of CA19-9 and CEA and various clinical characteristics of 112 patients were evaluated by the χ^2 test and Fisher's exact test. Survival analysis was conducted using the Kaplan-Meier survival and Cox proportional hazards model analysis. The difference in survival curves was evaluated with a log-rank test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Complications after the operation

In this study, no patients died during the operation. Forty-three patients developed one or more complications after the operation, with an incidence rate of 38.39% (43/112). The most common postoperative complications included pancreatic fistula, biliary fistula, intra-abdominal bleeding, gastric emptying disorders and peritoneal cavity infection.

Twenty-one patients developed postoperative pancreatic fistula, according to the diagnosis criteria of the ISGPF. There were 9 cases of Grade A pancreatic fistulas, 8 cases of Grade B pancreatic fistulas, and 4 cases of Grade C pancreatic fistulas. We also found that anastomosis of the pancreatic duct and jejunal mucous membrane and invaginated pancreaticoenterostomy had no influence on pancreatic fistulas. However, the incidence rate of pancreatic fistulas in patients with a pancreatic duct with a diameter greater than 4 mm was significantly lower than that in patients with a pancreatic duct with a diameter smaller than 4 mm. The incidence rate of postoperative biliary fistula was 1.78% (2/112). Seven patients developed a peritoneal cavity infection and 5 patients developed gastric emptying disorders.

After the operation, 2 patients needed reoperation, with an incidence rate of 1.78% (2/112). Of them, 4 patients received another laparotomy because of pancreatic fistulas and 2 patients underwent reoperation only because of intraabdominal bleeding. One patient experienced intraabdominal massive haemorrhage because of pancreatic fistulas, and although reoperation was performed, he still died.

Associations between serum levels of CA19-9 and CEA and various clinical characteristics

We characterized the serum levels of CA19-9 and CEA from 112 DPC patients. For serum levels of CA19-9, 73 (65.17%) were lower than 120 U/mL, defined as negative, with 39 (34.82%) positive. For serum levels of CEA, 96 (85.71%) were lower than 5 ng/mL, defined as negative, with 16 (14.29%) positive.

As in our clinical correlation studies, serum levels of CA19-9 and CEA were compared with DPC characteristics and risk factors (Table 1). The following analysis showed that serum levels of CA19-9 was associated with serum levels of CEA and drainage mode (the *P* values were 0.000 and 0.033, respectively); While serum levels of CEA was associated with serum levels of CA19-9 and differentiation of the tumour (the *P* values

Table 1 The serum levels of CA19-9 and CEA and clinical characteristics for 112 patients with duodenal papilla carcinoma

Characteristic	No.	Serum CA19-9		*P* value	Serum CEA		*P* value
		Negative	Positive		Negative	Positive	
Gender				0.350			0.807
Male	74	46	28		63	11	
Female	38	27	11		33	5	
Age (yr)				0.621			0.699
< 60	61	41	20		53	8	
> 60	51	32	19		43	8	
Duration (yr)				0.938			0.699
< 1	54	35	19		47	7	
> 1	58	38	20		49	9	
Serum CA19-9 (U/mL)							0.000
< 120	73				69	4	
> 120	39				27	12	
Serum CEA (ng/mL)				0.000			
< 5	96	69	27				
> 5	16	4	12				
Serum total bilirubin (μmol/L)				0.105			0.526
< 34.2	43	32	11		38	5	
> 34.2	69	41	28		58	11	
Bile pre-drainage				0.945			0.933
No	78	51	27		67	11	
Yes	34	22	12		29	5	
Tumour diameter (cm)				0.820			0.934
< 2	76	49	27		65	11	
> 2	36	24	12		31	5	
Pancreatic duct diameter (mm)				0.351			0.015
< 4	78	53	25		71	7	
> 4	34	20	14		25	9	
Drainage mode				0.033			0.928
Inside	85	60	25		73	12	
Outside	27	13	14		23	4	
End-to-end invagination				0.184			0.145
No	90	56	34		75	15	
Yes	22	17	5		21	1	
Blood loss (mL)				0.786			0.059
< 400	67	43	24		54	13	
> 400	45	30	15		42	3	
Delayed emptying				0.227			0.350
No	107	71	36		91	16	
Yes	5	2	3		5	0	
Pancreatic fistula				0.874			0.166
No	91	59	32		76	15	
Yes	21	14	7		20	1	
Differentiation				0.253			0.033
Well	40	24	16		37	3	
Moderate	38	23	15		28	10	
Poor	34	26	8		31	3	
T stage				0.946			0.062
T1	14	10	4		14	0	
T2	34	22	12		31	3	
T3	35	23	12		30	5	
T4	29	18	11		21	8	
N stage				0.078			0.301
N0	88	61	27		77	11	
N1	24	12	12		19	5	
TNM stage				0.682			0.109
ⅠA	14	10	4		14	0	
ⅠB	27	18	9		25	2	
ⅡA	28	20	8		24	4	
ⅡB	14	7	7		12	2	
Ⅲ							

were 0.000 and 0.033, respectively). The serum levels of CA19-9 and CEA were closely correlated (χ^2 = 13.277, r = 0.344, P = 0.000). No evidence of a significant association was observed between alteration of serum levels of CA19-9 or CEA and other characteristics or risk factors.

Associations between survival and various clinical characteristics

As of August 2015, we followed up all patients after the operation, with a median follow-up of 60 mo (ranging from 4 mo to 168 mo).There was a total of 52 patients with nodiseaseprogression, and of them, 48 patients lived longer than 5 years. A total of 60 patients died of this disease, with an median survival of 24.50 mo (ranging from 4 mo to 80 mo). The overall 5-year survival was 50.00% for 112 patients undergoing SPD (Figure 1A).

The Kaplan-Meier survival analysis showed that increased serum levels of CA19-9, CEA, and total bilirubin were correlated with a poor prognosis, as well as a senior grade of infiltration depth, lymph node metastases, and TNM stage(the *P* values were 0.033, 0.018, 0.015, 0.000, 0.000 and 0.000, respectively) (Tables 2 and 3, Figure 1B-F).

Only the senior grade of infiltration depth (T3/4) and TNM stage (ⅡB/Ⅲ) retained their significance when adjustments were made for other known prognostic factors in Cox multivariate analysis (*RR* = 2.211, *P* = 0.022 and *RR* = 2.109, *P* = 0.047).

DISCUSSION

The incidence rate of DPC is low, only accounting for 5% of gastrointestinal malignant tumours[11-13]. However, because the special position of duodenal papilla, *i.e.*, it is located at the opening of pancreatic duct, early DPC may manifest as painless progressive jaundice. SPD has always been the most important treatment mode of DPC. According to the literature, it had different 5-year survival rates and factors influencing survival[14,15]. Therefore, in this study, we carried out long-term follow-up and prognosis analysis of patients with DPC who received SPD in our centre to provide a theoretical basis for prognosis improvement of the patients.

Jaundice is the most common early clinical symptom in patients with DPC, and whether treatment for jaundice should be performed before the operation is always a topic of dispute in surgery. Some scholars think that preoperative high bilirubin may inhibit hepatocyte function and induce endotoxin dysmetabolism, thereby increasing the incidence rate of postoperative complications and influences the prognosis of patients[16,17]. However, some scholars have opposing views[18,19]. In this experiment, a poor survival was found in patients with increased bilirubin more than 34.2 μmol/L, with a 5-year survival rate of 40.6% (*P* = 0.015). Meanwhile, 34 patients who had placement of stents or drainage tubes before the operation, live longer than the other 78 patients who did not receive treatment for jaundice, with a 5-year survival rate of 55.9% *vs* 47.4%, although the difference was not significantly (*P* = 0.285). Based on these data, we proposed that increased bilirubin more than 34.2 μmol/L plays a bad role in the survival of patients with DPC after SPD.

Tumour markers are mainly used for the detection of primary tumours and the differentiation of benign and malignant tumours, and they have good clinical guidance significance for the judgement of the efficacy of tumour treatment and their occurrence and prognosis of tumours. There is still a lack of specific tumour markers for DPC. In our study, serum levels of CA19-9 was associated with drainage mode (*P* = 0.033); While serum levels of CEA was associated with tumour differentiation (*P* = 0.033). Besides, the serum levels of CA19-9 and CEA were closely correlated (*r* = 0.344, *P* = 0.000). A study by Dorandeu *et al*[20] demonstrated that preoperative serum levels of CA199 and CEA were negatively related to the prognosis of patients with DPC; however, our study has opposing views. Among the 112 patients with DPC, there were 39 patients with increased serum CA19-9 and 16 patients with increased serum CEA levels. When the patients with increased levels of serum CA199were compared with the others, the 5-year survival rates were significantly lower (38.3% *vs* 56.2%, *P* = 0.033). The same tendency was present in levels of serum CEA, with the 5-year survival rates of 25.0% *vs* 54.1% (*P* = 0.018). This indicated that the serum levels of CA19-9 and CEA are worth considering as the basis of diagnosis and prognosis for patients with DPC after SPD.

Pancreas-duodenum operations may cause great trauma, and there are many postoperative complications, of which pancreatic fistula is the most common complication[21,22]. In this study, the incidence rate of pancreatic fistula was 18.75% and was mainly Grade A and B pancreatic fistulas. However, we also found that whether there is a pancreatic fistula or not is not related with the long-term survival of patients (*P* = 0.500). In our study, we also investigated the influence of different factors on pancreatic fistulas, and our study found that anastomosis of the pancreatic duct and jejunal mucous membrane and invaginated pancreaticoenterostomy had no influence on pancreatic fistulas; different pancreatic duct drainage modes and whether Braun anastomosis was added also had no influence on the occurrence of pancreatic fistulas.

When it comes to the relationship between the size of tumour and the prognosis of patients, the study by Di Giorgio *et al*[23] demonstrated that among the 64 patients who underwent SPD, the prognosis of patients with tumours with a diameter greater than 2 cm was significantly inferior to those with tumours less than 2 cm. However, the results of our study were different; there was no significant difference in prognoses between the patients with tumours with a diameter greater than 2 cm and the others, and a diameter of 2 cm is not a boundary for the difference in prognosis.

We also found that the infiltration depth of tumours is an independent factor influencing the prognosis of patients after SPD. The study by Di Giorgio *et al*[4] demonstrated that for patients who underwent SPD

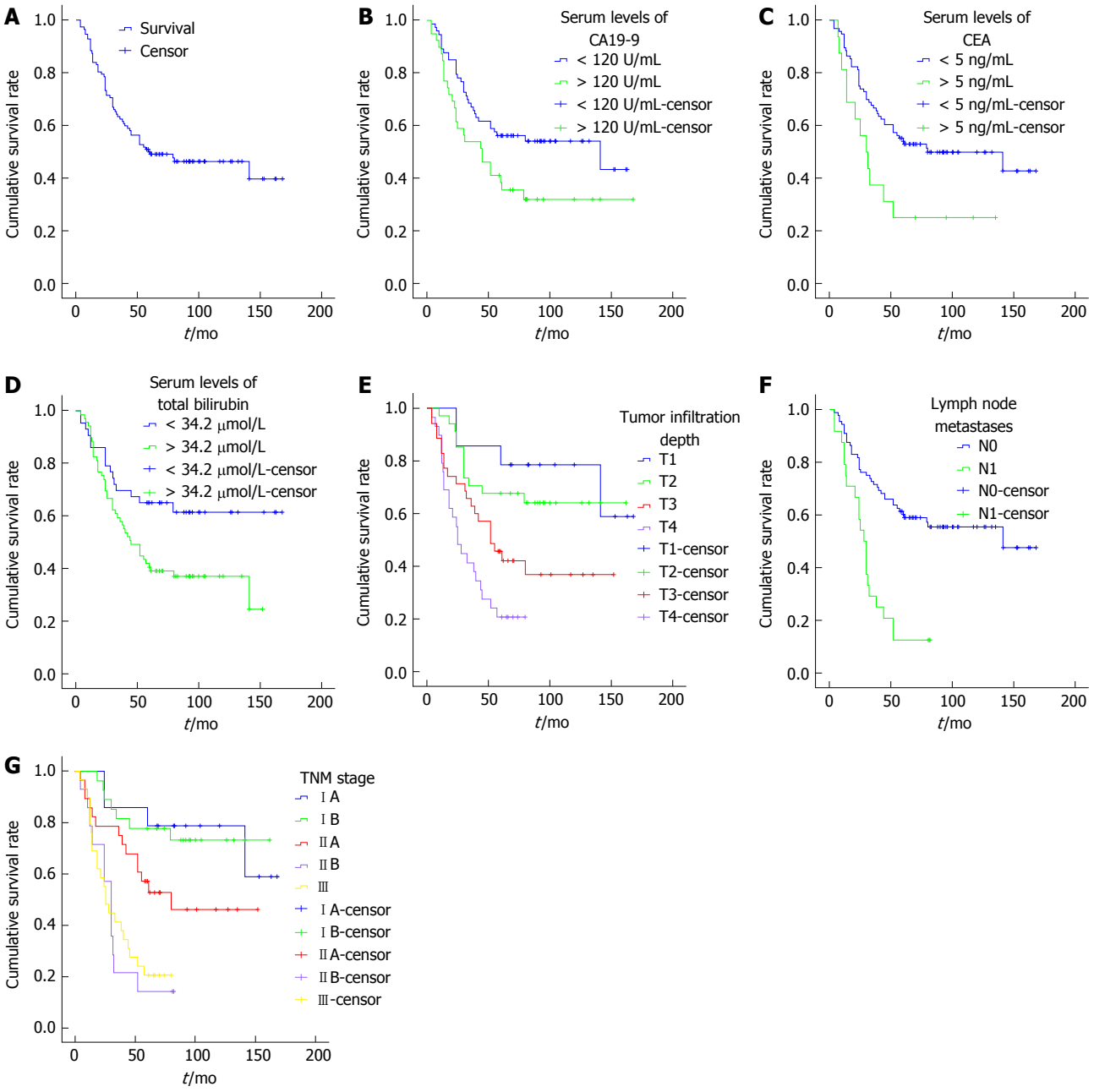

Figure 1 Kaplan-Meier plots show the association of survival and significant factors. A: The overall 5-year survival was 50.00%, with a median follow-up of 60 mo (ranging from 4 mo to 168 mo); B: Increased serum levels of CA19-9 was associated with decreased survival (P = 0.033); C: Increased serum levels of CEA was associated with decreased survival (P = 0.018); D: Increased serum levels of total bilirubin was associated with decreased survival (P = 0.015); E: The senior grade of infiltration depth was associated with decreased survival (P = 0.000), with significant difference between T1 and T3, T4 (P = 0.022, 0.000), as well as the difference between T2 and T3, T4 (P = 0.036, 0.000) and the difference between T3 and T4 (P = 0.049); F: The senior grade of lymph node metastases was associated with decreased survival (P = 0.000); G: TNM stage was associated with decreased survival (P = 0.000), with significant difference between Ⅰ A and Ⅱ B, Ⅲ (P = 0.000, 0.000), as well as the difference between Ⅰ B and Ⅱ B, Ⅲ (P = 0.000, 0.000) and the difference between Ⅱ A and Ⅱ B, Ⅲ (P = 0.006, 0.000).

because of duodenal ampulla tumours, the prognosis of patients with Stage T1/2 tumours was significantly better than that of patients with Stage T3/4 tumours. Our study also demonstrated that the infiltration depth of tumours has influence on the prognosis of patients; even when adjustments were made for other known prognostic factors in Cox multivariate analysis, the senior grade of infiltration depth (T3/4) and TNM stage (Ⅱ B/Ⅲ) retained their significance (RR = 2.211, P = 0.022 and RR = 2.109, P = 0.047).

Whether there are lymph node metastases is an important factor influencing the prognosis of malignant tumours. The study by Klein *et al*[24] reported that lymph node metastasis was a key factor influencing tumour recurrence and the survival of patients with ampullary carcinoma. Other studies demonstrated that the number of lymph node metastases was related to the prognosis of patients with periampullary carcinoma[25,26]. Our experiment led to the same conclusion; *i.e.*, the 5-year survival rate of patients

Table 2 Univariate analysis comparing overall survival to prognostic factors in 112 patients with duodenal papilla carcinoma

Factors	No.	5 years survival	χ^2	*P* value
Gender			0.561	0.454
Male	74	48.6%		
Female	38	52.4%		
Age (yr)			0.022	0.883
< 60	61	46.3%		
> 60	51	48.9%		
Duration (yr)			0.409	0.523
< 1	54	41.7%		
> 1	58	53.4%		
Serum CA19-9 (U/mL)			4.566	0.033
< 120	73	56.2%		
> 120	39	38.3%		
Serum CEA (ng/mL)			5.554	0.018
< 5	96	54.1%		
> 5	16	25.0%		
Serum total bilirubin (μmol/L)			5.929	0.015
< 34.2	43	65.1%		
> 34.2	69	40.6%		
Bile pre-drainage			1.144	0.285
No	78	47.4%		
Yes	34	55.9%		
Tumour diameter (cm)			0.185	0.667
< 2	76	51.3%		
> 2	36	47.2%		
Pancreatic duct diameter (mm)			0.493	0.483
< 4	78	51.2%		
> 4	34	47.1%		
Drainage mode			0.006	0.939
Inside	85	48.2%		
Outside	27	55.3%		
End-to-end invagination			0.592	0.442
No	90	48.9%		
Yes	22	54.5%		
Blood loss (mL)			0.052	0.820
< 400	67	49.2%		
> 400	45	51.1%		
Delayed emptying			0.614	0.433
No	107	50.5%		
Yes	5	40.0%		
Pancreatic fistula			0.455	0.500
No	91	47.1%		
Yes	21	57.1%		
Differentiation			3.676	0.159
Well	40	62.4%		
Moderate	38	39.5%		
Poor	34	47.1%		
Infiltration depth			22.424	0.000
T1	14	78.6%		
T2	34	67.6%		
T3	35	45.7%		
T4	29	20.7%		
lymph metastases			21.187	0.000
N0	88	60.2%		
N1	24	12.5%		
TNM stage			35.041	0.000
ⅠA	14	78.6%		
ⅠB	27	77.8%		
ⅡA	28	57.1%		
ⅡB	14	14.3%		
Ⅲ	29	20.7%		

with positive lymph node metastases was significantly lower than that of patients with negative lymph node metastases. However, lymph node metastases could not serve as an independent factor influencing the prognosis of patients after SPD.

In conclusion, for patients with DPC, the serum levels of CA19-9 and CEA were closely correlated, and play an important role in poor survival. Increased

Table 3 Multivariate analysis comparing overall survival to prognostic factors in 112 patients with duodenal papilla carcinoma

Factors	No.	5 years survival	Median survival (95%CI)(mo)	Relative risk (95%CI)	*P* value
CA19-9 (U/mL)					0.174
< 120	73	56.2%	141.0 (3.3-278.6)	1	
> 120	39	38.3%	45.0 (22.6-67.4)	1.550 (0.823-2.920)	
CEA (ng/mL)					0.528
< 5	96	54.1%	80.0 (10.4-149.5)	1	
> 5	16	25.0%	30.0 (18.2-41.8)	1.270 (0.605-2.663)	
Bilirubin (μmol/L)					0.264
< 34.2	43	65.1%		1	
> 34.2	69	40.6%	45.0 (29.9-60.1)	1.408 (0.772-2.566)	
Differentiation					0.137
Well	40	62.4%	141.0	1	
Moderate	38	39.5%	44.0 (3.09-57.1)	0.808 (0.399-1.635)	0.553
Poor	34	47.1%	52.0 (3.552-100.4)	1.636 (0.814-3.285)	0.167
Infiltration depth					0.022
T1 + T2	48	70.8%		1	
T3 + T4	64	34.4%	39.0 (26.3-51.7)	2.211 (1.119-4.367)	
lymph metastases					0.142
N0	88	60.2%	141.0	1	
N1	24	12.5%	28.0 (6.1-113.9)	1.744 (0.830-3.666)	
TNM stage					0.047
ⅠA + ⅠB + ⅡA	69	69.5%		1	
ⅡB + Ⅲ	43	18.6%	28.0 (21.6-34.4)	2.109 (1.010-4.406)	

serum levels of total bilirubin and lymph node metastases were also correlated with a poor prognosis. The senior grade of infiltration depth and TNM stage can serve as independent prognosis indexes in the evaluation of patients with DPC after SPD.

COMMENTS

Background

For duodenal papilla carcinoma (DPC), standard pancreaticoduodenectomy (SPD) is still the most important treatment. However, the prognosis assessment for DPC after SPD is not yet clear.

Research frontiers

Only a few researches have focused on the prognosis assessment for DPC after SPD. According to the literature, it had different 5-year survival rates and factors influencing survival.

Innovations and breakthroughs

In this study, the authors carried out long-term follow-up and prognosis analysis of patients with DPC who received SPD in our centre to provide a theoretical basis for prognosis improvement of the patients.

Applications

The senior grade of infiltration depth and TNM stage can serve as independent prognosis indexes in the evaluation of patients with DPC after SPD.

Peer-review

This study is very interesting. Over all, the study was well designed, and the manuscript is well written.

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P- Reviewer: Araujo RLC, Lee MW **S- Editor**: Wang JL
L- Editor: A **E- Editor**: Zhang FF

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5589-5601

DOI: 10.3748/wjg.v23.i30.5589

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

ORIGINAL ARTICLE

Clinical Trials Study

Efficacy and safety of Xiangsha Liujunzi granules for functional dyspepsia: A multi-center randomized double-blind placebo-controlled clinical study

Lin Lv, Feng-Yun Wang, Xiang-Xue Ma, Zhen-Hua Li, Sui-Ping Huang, Zhao-Hong Shi, Hai-Jie Ji, Li-Qun Bian, Bei-Hua Zhang, Ting Chen, Xiao-Lan Yin, Xu-Dong Tang

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Author contributions: Wang FY and Tang XD performed the research and drafted the manuscript; Li ZH, Huang SP and Shi ZH contributed to the development of the study protocol; Lv L contributed to the development of the study protocol and the drafting of the manuscript; Bian LQ, Zhang BH, Chen T and Yin XL recruited the participants; Ma XX and Ji HJ contributed to database management and statistical analysis; all authors approved the final version of the manuscript; Lv L took responsibility for the integrity of the work as a whole, from inception to published article.

Supported by the Major State Basic Research Development Program of China (973 Program), No. 2013CB531703; National Nature Science Foundation of China, No. 81503567 and No. 81673853; and the China Postdoctoral Science Foundation, No. 2015M1227 and No. 2016T90195.

Institutional review board statement: The study was reviewed and approved by the Ethic Committee of Xiyuan Hospital of China Academy of Chinese Medical Sciences.

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

Clinical trial registration statement: This study was registered at www.chictr.org.cn, and the registration identification number is ChiCTR-TRC-13003200. This study was also registered at https://clinicaltrials.gov/, and the registration identification number is NCT02762136.

Conflict-of-interest statement: The authors declare that they have no competing interests. Neither the funding agency nor any outside organization has a role in study design or manuscript preparation.

Data sharing statement: No additional data.

Manuscript source: Unsolicited manuscript

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Received: October 14, 2016
Peer-review started: October 19, 2016
First decision: December 2, 2016
Revised: May 12, 2017
Accepted: June 18, 2017
Article in press: June 19, 2017
Published online: August 14, 2017

Abstract

AIM

To assess the efficacy and safety of a Chinese herbal medicine (CHM), Xiangsha Liujunzi granules, in the treatment of patients with functional dyspepsia (FD).

METHODS

We performed a randomized, double-blind, placebo-controlled trial with patients from three centers. Two hundred and sixteen subjects diagnosed with FD according to ROME Ⅲ criteria and confirmed by upper gastrointestinal endoscopy and spleen-deficiency and Qi-stagnation syndrome were selected to receive Xiangsha Liujunzi granules or placebo for 4 wk in a 2:1 ratio by blocked randomization. The subjects also received follow-up after the 4-wk intervention. Herbal or placebo granules were dissolved in 300 mL of water. Participants in both groups were administered 130 mL (45 ℃) three times a day. Participants were evaluated prior to and following 4 wk of the intervention in terms of changes in the postprandial discomfort severity scale (PDSS) score, clinical global impression (CGI) scale score, hospital anxiety and depression scale (HADS) score, traditional Chinese medicine symptoms score (SS), scores of various domains of the 36-item short form health survey (SF-36), gastric emptying (GE) and any observed adverse effects.

RESULTS

Compared with the placebo group, patients in the CHM group showed significant improvements in the scores of PDSS, HADS, SS, SF-36 and CGI scale ($P < 0.05$ or $P < 0.01$). They also showed the amelioration in the GE rates of the proximal stomach and distal stomach ($P < 0.05$ or $P < 0.01$).

CONCLUSION

Xiangsha Liujunzi granules offered significant symptomatic improvement in patients with FD.

Key words: Functional dyspepsia; Chinese herbal medicine; Xiangsha Liujunzi; Efficacy; Randomized controlled trial

Core tip: Functional dyspepsia (FD) is a common clinical functional gastrointestinal disease with a very high incidence, seriously affecting the quality of life of patients. However, current chemical drugs do not achieve good curative effects. In the present study we performed a randomized, double-blind, placebo-controlled trial to assess the efficacy and safety of a Chinese herbal medicine, Xiangsha Liujunzi granules, in the treatment of patients with FD. We found that Xiangsha Liujunzi granules offered significant symptomatic improvement in patients with FD without adverse effects.

Lv L, Wang FY, Ma XX, Li ZH, Huang SP, Shi ZH, Ji HJ, Bian LQ, Zhang BH, Chen T, Yin XL, Tang XD. Efficacy and safety of Xiangsha Liujunzi granules for functional dyspepsia: A multi-center randomized double-blind placebo-controlled clinical study. *World J Gastroenterol* 2017; 23(30): 5589-5601 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5589.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5589

INTRODUCTION

Functional dyspepsia (FD), one of the most frequently encountered disorders of the gastrointestinal tract, is defined as the presence of symptoms originated in the epigastrium in the absence of any systemic, organic, or metabolic disease that is likely to explain the symptoms and is characterized by symptoms of meal-induced postprandial fullness or early satiety and upper abdominal discomfort or pain[1,2]. Previous studies have reported that the prevalence of FD varies considerably among different population-based studies, with percentages ranging from to 11% to 29%[3]. FD has symptoms similar to those of other conditions, including irritable bowel syndrome (IBS), peptic ulcer disease and gastroesophageal reflux. The two diagnostic categories of FD are (1) postprandial distress syndrome (PDS), which is characterized by the presence of meal-related early satiety and fullness; and (2) epigastric pain syndrome (EPS), which is characterized by the prominent symptom of epigastric pain that is generally not meal-related. However, evidence of their degree of overlap is mixed. Although its pathogenesis has not yet been fully clarified, abnormalities in gastric motor and sensory function and, more recently, low-grade duodenal inflammation have been identified[4,5]. The major pathophysiological mechanism underlying symptoms of FD is disorders of gastrointestinal motility[6]. Motility disorders lead to impaired gastric accommodation, myoelectric activity abnormalities and delayed gastric emptying (GE). Therefore, a prokinetic treatment approach is preferred in PDS. Recent evidence suggests that the subtypes of FD correlated with unhealthy dietary behaviors, especially skipping meals, eating extra meals and a preference to gas-producing food and sweet food[7]. It was recently found that the nutritional habits of FD patients are also a major factor in the production of discomfort symptoms. Carbonated drinks and fatty and spicy foods were the most common triggering food items in FD patients. Carbonated drinks and legumes were more likely to cause a symptom in PDS[8]. As the exact pathogenesis of FD has not yet been fully clarified, therapeutic drugs to relieve symptoms have thus not yet been established.

Symptomatic management of FD depends on the predominant symptoms and remains a substantial challenge. Treatment can include dietary

modifications, antiemetics, antispasmodics, prokinetics, antidepressants and analgesics, as well as proton pump inhibitors (PPIs)[9-12]. Despite the emergence of acotiamide in Japan[13], which has given hope to some FD patients, it does not benefit more patients. Therefore, the limited effectiveness of chemical drugs and the side effects of synthetic drugs make assessments of herbal preparations an appealing prospect[14]. It is becoming increasingly difficult to ignore the role of complementary and alternative medicine in the treatment of FD. Approaches based on complementary and alternative medicine in the treatment of FD have a long history, and several modalities have been investigated with some promising results[15]. A considerable amount of literature reported that at least 44 different herbal products have been recommended alone or in combination for the treatment of dyspeptic symptoms[16]. Complementary and alternative medicine consists of traditional Persian medicine (TPM), traditional Chinese medicine (TCM) and so on. Jollab, a TPM, appeared to be more effective than the placebo in patients with FD[17]. Adjuvant supplementation of honey-based formulation of *Nigella sativa* and the association between ginger and artichoke leaf extracts can cause significant symptomatic improvement in patients with FD[18,19]. Since Liujunzi decoction was proved to be effective against FD by a randomized controlled trial for the first time, a considerable amount of literature has been published on the treatment of FD with Chinese herbal medicine (CHM)[20,21]. To date, as the good efficacy of CHM for the relief of symptoms of FD, many dyspeptic patients chose CHM. Clinically, CHM is used in combination with Western medications or alone in China. The essence of TCM is treatment based on syndrome differentiation, which is based on the different conditions of each patient. At present, the phenomenon of the same drug treatment for the same disease with the same medicine is applied. Based on TCM theory, PDS can be categorized as "stuffiness and fullness", and the most related organ is the "spleen". A previous study has already demonstrated that the syndrome of "spleen-deficiency and Qi-stagnation" was the most common syndrome type in FD patients[22]. Based on these findings, the herbal preparation Xiangsha Liujunzi granules, a classical TCM formula, was designed and utilized in treating FD patients with "spleen-deficiency and Qi-stagnation" syndrome.

Despite ongoing clinical use, uncertainty remains regarding clinical efficacy and safety of CHM in the treatment of FD. The aim of this multicenter, double-blind, randomized placebo-controlled trial was to assess whether 4 wk of therapy with CHM was more efficacious than placebo in the relief of FD dyspeptic symptom and in improving quality of life[23]. We hoped to find strong evidence to support the use of CHM in PDS patients.

MATERIALS AND METHODS

Design

This study is a multicenter, randomized, double-blinded, placebo-controlled trial with two arms to examine the safety and efficacy of Xiangsha Liujunzi granules in the treatment of PDS patients. Participants were randomized into Xiangsha Liujunzi decoction or placebo groups in a 2:1 ratio. Since assigning an equal number of ill subjects to the ineffective placebo treatment was unethical, the 2:1 randomization plan was chosen to protect the rights of the subjects. The protocol was developed according to the Consolidated Standards of Reporting Trials statement[24] , Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) 2013[25] and SPIRIT 2013 explanation and elaboration: guidance for protocols of clinical trials[26]. The CHM group received Xiangsha Liujunzi granules, and the placebo group received a placebo. Then, all participants underwent a 4-wk treatment, followed by a 4-wk follow-up period. The study protocol was approved by the ethics committees of Xiyuan Hospital of China Academy of Chinese medical sciences, Wuhan Integrated TCM and Western Medicine Hospital, and Guangdong Province TCM Hospital, and informed consent was obtained from each participant. The study design is shown in Figure 1.

Subjects

Subjects were recruited from outpatients of three hospitals (Xiyuan Hospital of China Academy of Chinese Medical Sciences, Guangdong Province Traditional Chinese Medical Hospital, Wuhan Integrated TCM & Western Medicine Hospital) in China, between July 2013 and July 2016. Participants were recruited through advertising media, direct calls, and health promotion events. Advertisements were put on notice boards and homepages of the hospitals and local newspapers. Patients were required to provide their medical history, receive a physical examination and laboratory safety tests, and undergo a gastroscopy. Only those who fulfilled the Rome III criteria (Table 1) and TCM Diagnostic Criteria for Distention and Fullness (Table 2) of the principle for clinical research on new drugs of TCM[27] were considered eligible subjects. The inclusion and exclusion criteria are shown in Table 3. Written informed consent was obtained from all patients prior to inclusion in the trial. Participants were free to withdraw from the study at any time.

Randomization and blinding

The participants in the three hospitals were randomly assigned to two groups using block randomization. Random numbers based on the allocation sequence were generated using SAS (Version 9.2, Channel-leadian Pharmaceuticals R&D, Co., Ltd.; Haidian District, Beijing) by an independent statistician at the Good Clinical Practice (GCP) center of Xiyuan

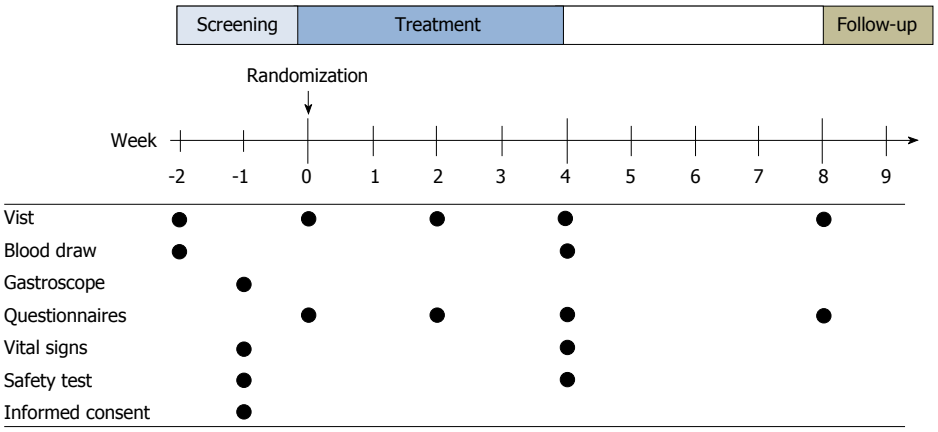

Figure 1 Study design. There was a screening period of 2 wk before randomization. Blood draw, gastroscopy, and physical examination were performed and written informed consent were obtained from all patients in this period. Study visits were arranged at -2 wk, 0 wk, 2 wk, 4 wk and 8 wk. Some questionnaires were performed by phone during the treatment period, and this was followed by a 4-wk follow-up period.

Table 1 Rome III diagnostic criteria for functional dyspepsia

Functional dyspepsia	Subtype
The last 3 mo with symptom onset at least 6 mo before diagnosis, and must include:	Postprandial distress syndrome
1 One or more of:	Must include one or both of the following:
a, Bothersome postprandial fullness	1 Bothersome postprandial fullness, occurring after ordinary sized meals, at least several times per week
b, Early satiation	2 Early satiation that prevents finishing a regular meal, at least several times per week
c, Epigastric pain	Supportive criteria
d, Epigastric burning	1 Upper abdominal bloating or postprandial nausea or excessive belching can be present
AND	2 EPS may coexist
2 No evidence of structural disease (including at upper endoscopy) that is likely to explain the symptoms	

Table 2 Traditional Chinese medicine diagnostic criteria for stuffiness and fullness

	Spleen-deficiency and Qi-stagnation
Primary symptoms	Abdominal distension, with obviously postprandial distress
	Anorexia or little appetite
Secondary symptoms	Nausea; acid reflux/heartburn; loose stools; fatigue/exhausted
	Fat tongue with slightly white coating
	Weak pulse

The diagnosis will be established when the participant conforms to all primary symptoms and any two of the secondary symptoms.

Hospital. Each randomization number was placed in a sequentially numbered opaque envelope that was sealed by the clinical research coordinator. After screening, the clinical investigator assigned the treatment according to the randomization number. Participants, clinical investigators, statisticians, and all related study staff were blinded. The blinding procedure was also verified by the authorized contract research organization. Group assignments were not revealed until the entire study was completed except in emergency situations if practical intervention was compulsory for further management of the participant.

Sample size

The trial was powered for superiority testing for the primary point. According to our preliminary studies, the effective rate of the Xiangsha Liujunzi decoction in treating FD was between 67% and 80%, and we estimated that the average effective rate was 70%, whereas that of the placebo for FD was approximately 45%[28]. A one-sided test yielding a 2.5% significance level was used to prove the hypothesis that Xiangsha Liujunzi granules is more effective than placebo for treating PDS. The patients were assigned to either the CHM group or the placebo group in a 2:1 ratio. The sample size was based on the superiority design as follows:

$$n_1 = \frac{[U_\alpha\sqrt{\pi_c(1-\pi_c)(1+c)/c} + U_\beta\sqrt{\pi_1(1-\pi_1)+\pi_2(1-\pi_2)/c}]^2}{(\pi_1-\pi_2)^2}$$

$n_2 = cn_1$

n_1: placebo; n_2: CHM

$\pi_c = (\pi_1 + c\pi_2)/(1 + c)$, $u_\alpha = 1.645$, $u_\beta = 1.985$, $c = 2$, $\pi_1 = 0.5$, $\pi_2 = 0.75$

The calculation indicated that a sample size of 180 would be sufficient (n = 120 in the CHM group, n =

Table 3 Inclusion and exclusion criteria

Inclusion criteria
1 Age 18-70 yr, Chinese reading and writing ability
2 Meeting the Rome III diagnostic criteria for PDS
3 Having the symptom of "spleen-deficiency and Qi-stagnation", "spleen-deficiency and damp-obstruction", "spleen-Yang deficiency".
4 Signing the informed consent form
Exclusion criteria
1 Combined with GI ulcer, erosive gastritis, atrophic gastritis, severe dysplasia of gastric mucosa or suspicious malignant lesion
2 Having overlap syndrome combined with gastroesophageal reflux disease or irritable bowel syndrome
3 Having alarm symptoms (weight loss, black or tar stool, dysphagia, *etc.*)
4 Having serious structural disease (disease of heart, lung, liver or kidney) or mental illness
5 History of surgery related with the gastrointestinal tract, except for appendectomy more than six months ago
6 Taking drugs which may affect the gastrointestinal tract, such as nonsteroidal anti-inflammatory drugs and aspirin
7 Allergy to the experimental medication
8 Difficulties in attending the trial (paralysis, serious mental illness, dementia, renal diseases, stroke, coronary atherosclerotic heart diseases, diabetes or mental diseases, illiteracy, *etc.*)
9 Pregnant or breastfeeding
10 Refusing to sign the informed consent form

Table 4 Components of the Xiangsha Liujunzi granules

Scientific name	Part used	Proportion of ingredients (100%)
Astragalus mongholicus	Root	12
Codonopsis pilosula	Root	12
Rhizoma Atractylodis Macrocephalae	Rhizome	12
Poria cocos	Sclerotium	12
Fructus Aurantii	Fruit	12
Amomum villosum	Fruit	6.4
Ligusticum chuanxiong Hort	Sclerotium	9.6
Rhizoma corydalis	Rhizoma	9.6
Medicated Leaven	Fermentation products	12
Glycyrrhiza uralensis Fisch	Root	2.4

60 in the control group). To allow for a 20% rate of dropouts and missing data, we recruited 144 patients for the CHM group and 72 patients for the placebo group.

Intervention

Qualified patients received a unique treatment number, which was fixed throughout the duration of the trial. The medication allocation was based on the treatment number. The composition and action of the herbal preparation of Xiangsha Liujunzi granules are summarized in Table 4. The placebo granules were made from dextrin (80%), rice (15%), bitter principle (5%) and coloring matter to ensure that the color, smell, taste and texture were similar to those of Xiangsha Liujunzi granules. Patients were informed to dissolve a sachet of granules (14 g) in 130 mL of hot water and to take the solution orally three times daily 1 h after breakfast, lunch and dinner for 4 wk. Both the herbal formula and placebo granules were manufactured by Beijing Tcmages Pharmaceutical Co., Ltd. (Shunyi District, Beijing) according to the standards of good manufacturing practice. All medications were packed identically in opaque aluminum bags with the same labeling form except the treatment number.

Ethical approval and registration

This trial was conducted according to the standards of the International Committee on Harmonization on GCP and the revised version of the Declaration of Helsinki. Institutional review boards at Xiyuan Hospital of China Academy of Chinese Medical Sciences, Wuhan Integrated TCM & Western Medicine Hospital, and Guangdong Province TCM Hospital approved this protocol. This trial was registered in the ChiCTR (ChiCTR-TRC-13003200, 19 April 2013) and Clinical Trials.gov (NCT02762136).

Outcomes

Primary outcome: We assessed FD symptoms using the change in postprandial discomfort severity scale (PDSS) from baseline (week 0) to the treatment endpoint (week 8).

PDSS: Based on Rome III criteria and past clinical trials[29], PDSS was designed to evaluate dyspeptic symptoms, including postprandial fullness, early satiety, upper abdominal pain or discomfort, and heartburn. The severity and frequency of each symptom are separately evaluated by a defined numerical score. The severity was scored as follows: 0, absent; 1, mild (awareness of symptoms but easily tolerated); 2, moderate (interference with normal activities); and 3, severe (incapacitating). The total severity score ranged from 0 to 12. The frequency scoring used the following scale: 0, absent (less than once per month); 1, rarely (less than once per week); 2, occasionally (less than three times per week); and 3, often (more than or three times per week). The total frequency score ranged from 0 to 12.

Secondary outcomes

Changes in the clinical global impression (CGI) scale score, TCM symptom score (SS), MOS 36-Item Short-

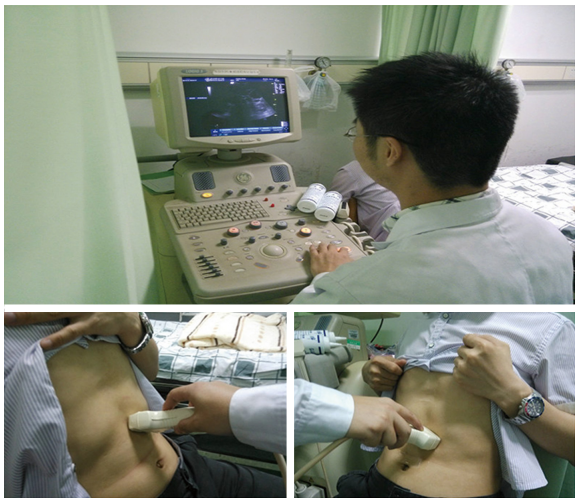

Figure 2 Gastric ultrasonography.

Form Health Survey (SF-36) score, hospital anxiety and depression scale (HADS) score, and GE were considered secondary outcomes.

CGI scale: The CGI scale[30] comprises two parts: CGI-severity (CGI-S) and CGI-improvement (CGI-I). The former evaluates the severity of psychopathology from 1 to 7, and the latter assesses the change from the initiation of treatment on a similar seven-point scale. In CGI-S, clinical investigators need to answer one question: "Considering your total clinical experience with this particular population, how mentally ill is the patient at this time?", which is rated on the seven-point scale: 1 = normal, not at all ill while 7 = the most extremely ill. Similarly, in CGI-I, clinical investigators need to answer another question: "Compared to the patient's condition at admission to the project, how is this patient's condition?" which is also rated on a seven-point scale: 1 = very much improved since the initiation of treatment; 4 = no change from baseline; 7 = very much worse since the initiation of treatment.

TCM SS: TCM SS is a scale utilized to evaluate the participant's discomfort of PDS based on TCM theory. SS contains 15 items of TCM terminology, which are used to assess the presence (yes/no) and severity of physical discomfort (0 = absent; 1 = mild; 2 = moderate; 3 = severe).

SF-36: SF-36, the most commonly used tool to assess quality of life that contains 36 items in eight dimensions, was used to assess the effect of experimental medication. The eight dimensions consist of general health (GH), physical functioning (PF), role physical (RP), role emotional (RE), social functioning (SF), bodily pain (BP), vitality (VT) and mental health (MH)[31].

HADS: The HADS, first developed by Snaith and Zigmond in 1983, was usually used to identify cases (possible and probable) of anxiety disorders and depression among patients in non-psychiatric hospitals[32-34]. The scale consists of 14 items, 7 related to anxiety (HADS-A) and 7 related to depression (HADS-D), each scored between 0 and 3. The authors of this scale recommended that if an individual had a score of 8, he/she should be regarded as a possible case. It was found that this threshold was optimal for HADS-A and HADS-D in both the general population and patients with somatic symptoms.

GE: GE was observed using gastric ultrasound[35]. Participants were asked to fast overnight before the ultrasonography. A 1120 mL esculent liquid was used as the test meal and was prepared by mixing 50 g of nutrient Cola Cao (enteral nutritional solid beverage, chocolate; Tianjin Cola Cao Food Co., LTD, Tanjin, China) and 100 g of milk powder (Nestle whole milk powder; Shuangcheng Nestle Co., Ltd, Heilongjiang, China) with 1100 mL of warm water, and the entire test meal volume was 1120 mL. This esculent liquid contained 26.5 g of protein, 29.1 g of fat and 75.5 g of carbohydrate (840 kcal). This mixed liquid meal consisted of 13% proteins, 48% carbohydrates and 39% lipids and was used at a caloric density of 0.75 kcal/mL. The liquid test meal was administered orally as tolerated at a fixed rate of 50 mL/min, and all participants were allowed to sit stationary on a chair for approximately 5 min while drinking this test meal. The subjects were scanned in a half-sitting position, sitting on an examining chair and leaning back at an angle of approximately 120°. Subjects were instructed not to move and to hold their breath at the end of expiration to permit diaphragmatic rising and restoration of the gastric configuration (Figure 2).

The proximal stomach and distal stomach volumes of each subject were scanned at six time points, which were fasting, maximum satiety, and 30 min, 60 min, 90 min, and 120 min after beginning ingestion.

GE rate was calculated as follows: (Amax - A)/Amax × 100 (%), where Amax is the volume of maximum satiety after the meal in the proximal stomach or distal stomach, and A is the volume at other time points after the meal in the proximal stomach or distal stomach.

Safety monitoring

To assess the safety of the 4 wk treatment, routine tests of blood, urine and stool samples, as well as an electrocardiogram and blood biochemical tests (ALT, AST, BUN, Scr) were conducted before randomization and immediately after the completed treatment. During the trial, any adverse events were recorded in detail and documented using case report forms, and appropriate treatment was provided to the participant immediately if necessary.

Statistical analysis

All statistical analyses were conducted in a blind

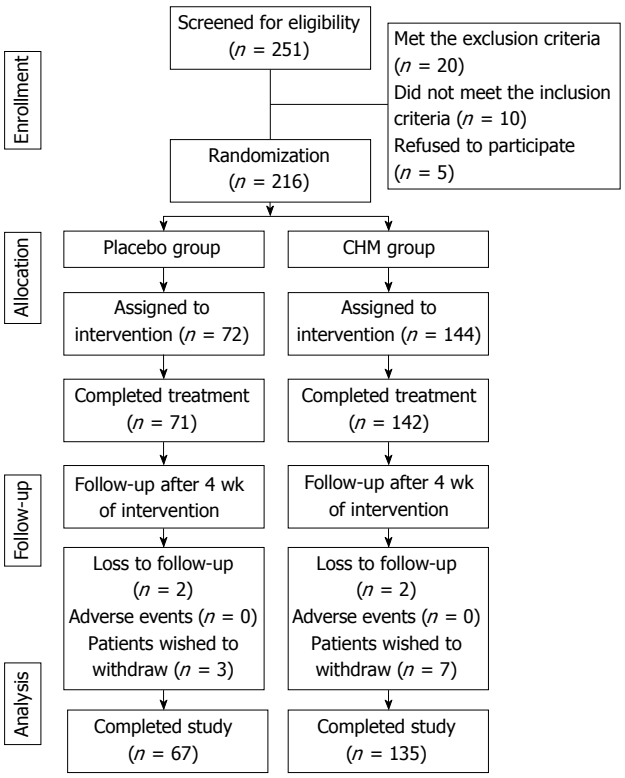

Figure 3 Flow of participants in the study.

Table 5 Subject characteristics (n = 202) n (%)

Characteristic		Placebo group (n = 67)	CHM group (n = 135)
Gender	Female	86 (63.7)	43 (64.2)
Age	mean ± SD	43.89 ± 13.32	44.33 ± 12.41
	20-29	12 (17.9)	21 (15.6)
	30-39	18 (26.9)	28 (20.7)
	40-49	8 (11.9)	31 (22.9)
	50-59	17 (25.4)	28 (20.7)
	60+	12 (17.9)	21 (15.6)
Level of education	Primary school	5 (7.5)	9 (6.7)
	Junior school	6 (8.9)	22 (16.3)
	Senior school	15 (22.4)	23 (17)
	Degree or above	41 (61.2)	81 (60)

CHM: Chinese herbal medicine.

manner by an independent statistician using SPSS (Version 19.0; SPSS Inc, Chicago, IL, United States). Significance was defined as a 2-sided P value of < 0.05. Demographic, clinical, and outcome variables were described using mean ± SD for continuous variables and percentages for categorical variables. First, the baseline characteristics of both groups were compared, including gender, age, and level of education. Second, the efficacy of CHM and placebo was compared, including the primary outcome and all secondary outcomes. All analyses in this study were based on the intention-to-treat principle. Missing values were recorded by the last-observation-carried-forward method, and this analysis imputes the last measured value of the endpoint to all subsequent scheduled but missing evaluations. The baseline characteristics were compared using either χ^2 or the Student's t-tests. Primary and secondary outcomes are presented as the mean and SD and were analyzed using independent t-tests and Wilcoxon signed-rank tests. Adverse events were calculated and compared using χ^2 test.

RESULTS

Study population

Between July 2013 and July 2016, a total of 216 patients were recruited: 144 were randomized into the CHM group, and 72 were randomized into the placebo group. Fourteen patients withdrew from the trial due to a lack of efficacy (9 patients in the CHM group and 5 patients in the placebo group). No adverse events were reported. The physiological tests obtained after 4 wk of treatment showed no abnormal values.

Participant flow

The flow of participants in the study is summarized in Figure 3.

Baseline data

The general characteristics of the patients are shown in Table 5. No significant differences were identified between the two groups in parameters such as gender, age and level of education.

Primary outcome

PDSS: At week 2, the symptom score of postprandial fullness in the CHM group was significantly better than that in the placebo group (t = 3.561, P = 0.000). After 4 wk of treatment, the scores of postprandial fullness, early satiety and epigastric pain of PDSS assessed by patients were significantly better in the CHM group than in the placebo group (t = 3.976, P = 0.000; t = 5.302, P = 0.000; t = 2.077, P = 0.039). These three symptom scores were also significantly better in the CHM group than in the placebo group during the follow-up period (t = 3.336, P = 0.001; t = 6.658, P = 0.000; t = 2.244, P = 0.026). The results were clinically meaningful (Figure 4).

Secondary outcomes

CGI scale: After 4 wk of treatment, the ratings of the CGI showed the following significant results for the CHM group *vs* placebo group: very much improved (5.9% *vs* 3.0%), much improved (36.3% *vs* 25.4%), slightly improved (44.4% *vs* 44.8%), and unchanged or deteriorated (11.8% *vs* 22.4%) (Z = -2.244, P = 0.025). The ratings of the CGI between the CHM group and placebo group during the follow-up period were as follows: very much improved (5.2% *vs* 3.0%), much improved (40.0% *vs* 22.4%), slightly improved (40.7% *vs* 46.3%), and unchanged or deteriorated (13.3% *vs* 25.4%) (Z = -2.054, P = 0.04). The results were

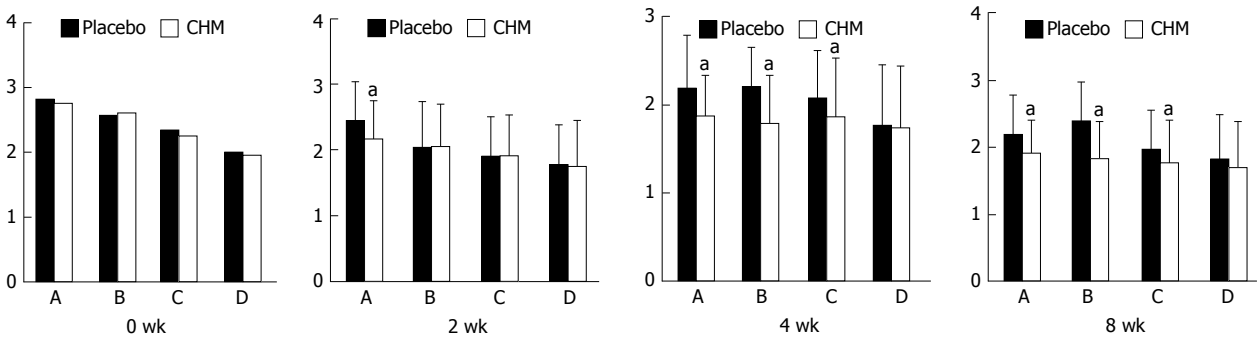

Figure 4 Postprandial discomfort severity scale. A: Postprandial fullness; B: Early satiety; C: Epigastric pain; D: Epigastric burning. [a]*P* < 0.05 *vs* placebo group. CHM: Chinese herbal medicine.

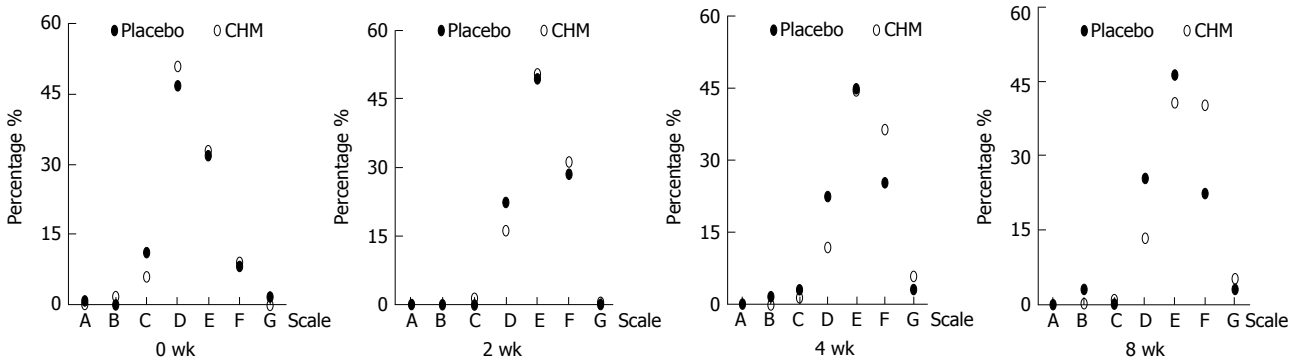

Figure 5 Clinical global impression scale. A: Very much worse since the initiation of treatment; B: Worse since the initiation of treatment; C: Slightly worse since the initiation of treatment; D: No change from baseline; E: Slightly improved since the initiation of treatment; F: Significantly improved since the initiation of treatment; G: Very much improved since the initiation of treatment. CHM: Chinese herbal medicine.

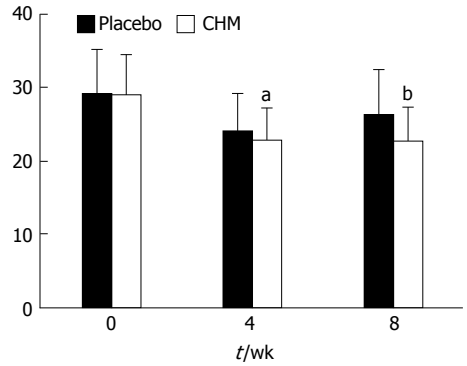

Figure 6 Traditional Chinese medicine symptom scores. [a]*P* < 0.05, [b]*P* < 0.01 *vs* placebo group. TCM: Traditional Chinese medicine; CHM: Chinese herb medicine.

clinically meaningful (Figure 5).

TCM SS: There was no significant difference between the two groups regarding SS at baseline (t = -0.173, P = 0.863). After 4 wk of treatment, SS in the CHM group was significantly better than that in the placebo group (t = -2.067, P = 0.04). The same result was also found during the follow-up period (t = -4.752, P = 0.000) (Figure 6).

SF-36: There was no significant difference between the two groups regarding the eight dimensions of GH, PF, RP, RE, SF, BP, VT and MH in the SF-36 at baseline (t = -1.33, P = 0.185; t = -1.44, P = 0.151; t = 0.787, P = 0.432; t = 1.248, P = 0.214; t = 0.267, P = 0.79; t = 0.631, P = 0.529; t = -1.965, P = 0.051; t = -0.372, P = 0.71). After 4 wk of treatment, the SF-36 scores in the CHM group were significantly better than those in the placebo group (P < 0.05 or P < 0.01). The same result was also found during the follow-up period (P < 0.05 or P < 0.01) (Figure 7).

HADS: There was no significant difference between the two groups regarding HADA and HADD (t = 0.016, P = 0.987; t =-0.400, P = 0.690) at 0 wk. After 4 wk of treatment, the HADA and HADD scores in the CHM group were significantly better than those in the placebo group (t = -2.179, P = 0.032; t = -2.429, P = 0.017). The same result was also found during the follow-up period (t = -2.76, P = 0.006; t = -3.646, P = 0.000) (Figure 8).

GE: The GE rate of the proximal stomach (GERPG) in the CHM group at various time points was higher than that of the control group, and the differences were significant (P < 0.01). Moreover, the gastric emptying rate of the distal stomach (GERDG) in the CHM group, compared with the control group, decreased at 30 min

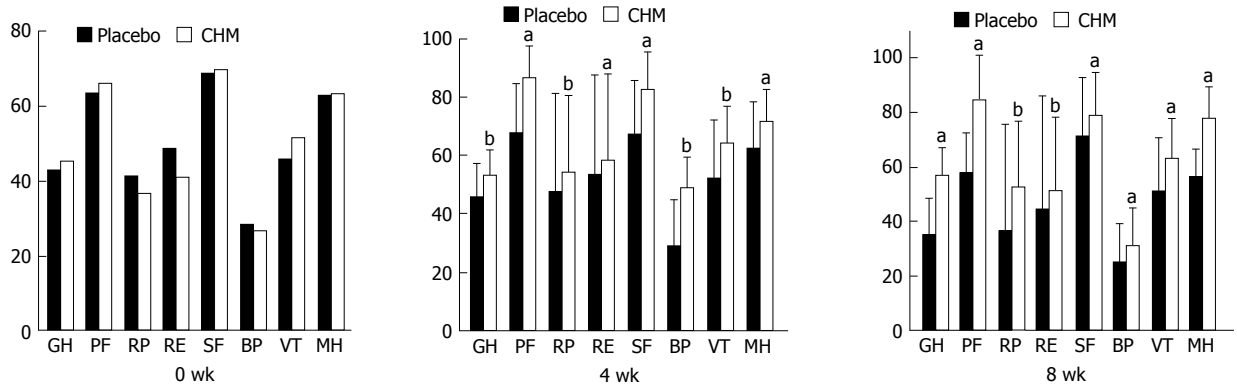

Figure 7 MOS 36-item short-form health survey. GH: General health; PF: Physical functioning; RP: Role physical; RE: Role emotional; SF: Social functioning; BP: bodily pain; VT: Vitality; MH: Mental health. [a]*P* < 0.05, [b]*P* < 0.01 *vs* placebo group. CHM: Chinese herb medicine.

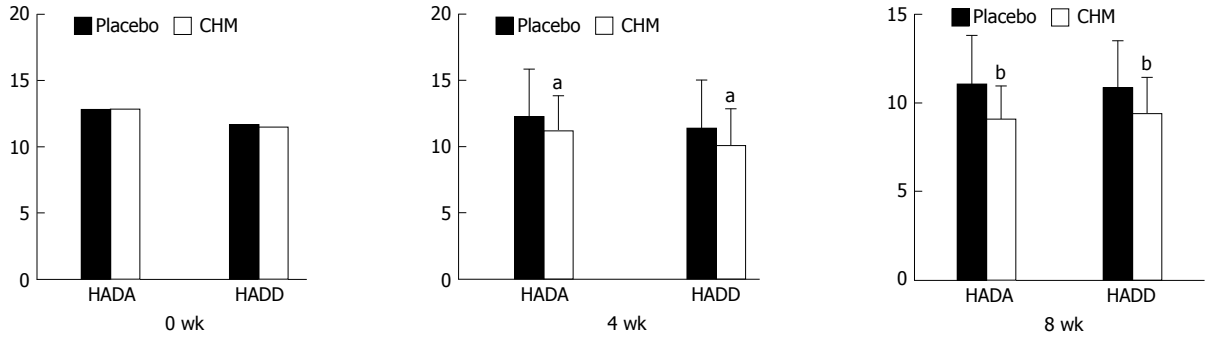

Figure 8 Hospital anxiety and depression scale. [a]*P* < 0.05, [b]*P* < 0.01 *vs* placebo group. CHM: Chinese herb medicine; HADA: Hospital anxiety and depression scale-Anxiety; HADD: Hospital anxiety and depression scale-Depression.

after the meal but increased at the other time points, and the differences were significant ($P < 0.05$ or $P < 0.01$) (Table 6).

Adverse effects: In the 4 wk treatment and the follow-up period, all subjects had no adverse effects.

DISCUSSION

FD is a common gastrointestinal disease. The prevalence of FD is 20%-25% in Western countries and 8%-23% in Asia[36,37]. In China, approximately 10% of general outpatients have dyspepsia, which account for 50% of digestive internal medicine clinic services. FD involves various pathophysiological disturbances and many pathogenic factors, including impaired accommodation, delayed GE, and hypersensitivity to gastric distention. Several studies have reported that visceral hypersensitivity is the mechanism of postprandial abdominal bloating and distension. The normal abdominal accommodation is altered by an abnormal viscerosomatic response to meal ingestion[38]. Patients with PDS have the characteristics of fasting and postprandial hypersensitivity, while patients with EPS have the characteristics of a reduction in gastric compliance[39]. There are multiple drug options in clinical practice, including PPIs, prokinetic agents, anti-*Helicobacter pylori* drugs, antidepressant drugs and mucosal protective agents. Because the pathophysiology of FD involves several physiological systems and a variety of treatment classes are available, health care providers face uncertainty in selecting therapies for patients with FD. Furthermore, these chemical drugs are not satisfactory or effective. Therefore, many patients with FD seek treatment with complementary and alternative medicine[15]. TCM, as an important component of complementary and alternative medicine, has attracted increasing attention in the world, especially in the treatment of functional gastrointestinal diseases.

Based on TCM theory, the "spleen" governs movement and transformation of food and fluid. Emotional injury, food or drink, and congenital defects are main pathogenic factors of a functional disorder of the "spleen", which can lead to the stagnation of Qi and dampness and then result in "stuffiness and fullness". As spleen deficiency is the essence of these three types of syndrome, invigorating the spleen is the critical principle of treatment[40,41]. This pathogenic factor causes abnormal function of the upper abdomen, spleen and stomach, resulting in the presence of abdominal distension, appetite disorder, loose stools, and lassitude. The TCM pathogenesis of FD is spleen deficiency and Qi-stagnation. Xiangsha Liujunzi decoction is a classical formula based on invigorating the spleen and has been used by TCM

Table 6 Gastric emptying rates of proximal and distal stomach

Time	Placebo (n = 67)	CHM (n = 135)	t	P value
0 wk				
GE rate of proximal stomach				
30 min	32.85 ± 19.27	30.91 ± 16.67	0.653	0.515
60 min	49.26 ± 18.67	49.46 ± 17.74	-0.066	0.947
90 min	66.37 ± 14.63	63.72 ± 17.51	0.948	0.344
120 min	76.54 ± 13.97	75.39 ± 13.91	0.487	0.627
GE rate of distal stomach				
30 min	15.68 ± 35.26	6.81 ± 37.61	1.576	0.117
60 min	32.49 ± 30.82	26.91 ± 33.36	1.124	0.263
90 min	49.26 ± 25.93	41.84 ± 33.59	1.555	0.122
120 min	61.61 ± 21.85	57.52 ± 27.02	1.053	0.294
4 wk				
GE rate of proximal stomach				
30 min	28.48 ± 13.13	36.31 ± 10.78	-4.195	0.000
60 min	47.21 ± 16.76	56.92 ± 10.27	-4.722	0.000
90 min	59.59 ± 17.71	72.92 ± 16.94	-6.919	0.000
120 min	71.47 ± 16.94	82.31 ± 7.35	-5.888	0.000
GE rate of distal stomach				
30 min	7.03 ± 35.40	-24.34 ± 34.02	5.707	0.000
60 min	26.94 ± 31.39	40.83 ± 16.37	-3.871	0.000
90 min	50.06 ± 21.97	56.90 ± 15.35	-2.406	0.017
120 min	61.89 ± 15.26	67.66 ± 13.41	-2.578	0.011

CHM: Chinese herb medicine; GE: Gastric emptying.

doctors for hundreds of years. In the past two decades, a considerable number of clinical trials have proved the efficacy and safety of this decoction in the treatment of FD[20,34,42,43]. Currently, no patient-reported outcome instrument is available for the evaluation of treatment efficacy in PDS, which makes the evaluation of treatment effects in FD difficult. To improve the evaluation criteria of this study, we chose the PDSS, CGI, SS, SF-36, and HADS. CGI instrument, an established outcome measure in psychopharmacology research, is a reliable measure of fatigue[44]. Symptoms of fatigue are often part of the clinical presentation of spleen deficiency. Mental and psychological disorders are one of the pathogenic factors for FD. Clinically, we found many FD patients with anxiety and depression symptoms. One of the "gold standard" to assess the efficacy of antidepressants and anti-anxiety medications is the Hamilton rating scale. Another scale to assess the efficacy of antidepressants and anti-anxiety medications is the CGI, which has similar effect sizes to the HAM rating scale[45]. Therefore, CGI instrument has been used in clinical practice as well as research. Zigmond and Snaith devised the HADS, which has the advantages of simplicity, fastness and ease of use, to assess anxiety and depression in a general medical population 33 years ago[46]. This scale has recently been used to assess FD patients with anxiety and depression. The remarkable placebo response is the most difficulty in clinical trials on FD patients. In order to ensure that the participants could not be able to make a distinction between placebo and active treatment, we tried our best to make the drugs in the two groups indistinguishable for the participants. The placebo granules were made from 80% dextrin, 15% rice, 5% bitter principle and coloring matter to ensure that the color, smell, taste and texture were similar to those of Xiangsha Liujunzi granules. In this clinical study, we found that Xiangsha Liujunzi granules can relieve the postprandial fullness, early satiety, and epigastric pain symptoms in PDS patients, but the improvement in the epigastric burning sensation was not superior to that achieved by the placebo. This result may be because the inhibition of gastric acid secretion by CHM was not better than that of PPIs. The SF-36 scale was used to evaluate the improvement in quality life of patients. Our results showed the advantage of the CHM compared to placebo, with a significant improvement of 8 subscale scores of GH, PF, RP, RE, SF, BP, VT, MH on the SF-36 at 2 wk and 4 wk. These results persisted until the end of the study (8 wk). The pathophysiology of FD is diverse and its symptoms are non-specific; the pathogenesis of FD is also related to mental and psychological factors. Therefore, some antidepressants have been used to treat FD, such as mirtazapine[47]. According to our results, the HADA and HADD scores were both reduced in the CHM group compared to the placebo group at 4 wk and 8 wk, and there was a significant difference between the two groups. The CHM treatment had significant improvement in gastrointestinal-specific anxiety and depression. This study also showed that TCM symptom scores significantly improved from 2 wk to 4 wk with CHM treatment, and the result persisted to the follow-up time (8 wk). This finding shows that TCM treatment can reduce the recurrence of FD symptoms. Finally, after 4 wk of CHM treatment, the number of participants with very much improved and much improved scores on the CGI scale, compared to placebo, was significantly higher at 4 wk.

FD is one of the most common gastrointestinal

diseases, accounting for up to 5% of outpatients in gastroenterology department[48]. GE of patients with FD is usually delayed in 30%-50%[49], including both liquid emptying[50] and solid emptying[48]. Food ingestion can directly aggravate dyspeptic symptoms of patients with FD. Data from questionnaires revealed that 75% of patients with FD had experience of aggravation of dyspeptic symptoms after ingestion of a meal. Previous studies had reported that 90% of patients with FD had aggravation of dyspeptic symptoms after ingestion of a standardized 250-kcal meal, and maximum symptom severity occurred between 45 and 90 min after meal[51]. In this study, we calculated the GERPG and GERDG at 4 time points and found that the GERDG of the CHM group at 30 min after a meal was negative. As we know, the proximal stomach functions as a reservoir, and the liquid food flows into the distal stomach and enlarges the volume of the distal stomach over time. This factor caused an illusion that the GERDG of the CHM group was decreased. Compared with those of the placebo group, the GERPG and GERDG of the CHM group were increased at 60 min, 90 min, and 120 min after the meal. The results of this study suggest that the curative effect of Xiangsha Liujunzi decoction can promote GE of patients with PDS. Furthermore, throughout the course of the experiment, participants were not found to have any adverse events.

Conclusion

We have demonstrated the clinical efficacy of the Xiangsha Liujunzi granules to improve the symptomatic symptoms of patients with FD. In this randomized, double-blinded, placebo-controlled trial, Xiangsha Liujunzi granules was shown to be effective in the management of FD, especially in patients with post-prandial fullness and bloating, early satiety, and epigastric pain. This treatment will provide a new option for clinicians in treating PDS. However, the mechanism of action for CHM to reduce gastrointestinal symptoms is still unknown, and further studies are needed to determine the precise mechanisms of action.

COMMENTS

Background

Functional dyspepsia (FD), one of the most common functional gastrointestinal disorders, has two diagnostic categories that are postprandial distress syndrome (PDS) and epigastric pain syndrome. Its prevalence ranges from 11% to 29%, and it seriously affects the quality of life of patients. However, current chemical drugs do not achieve good curative effect, and it is therefore necessary to find a new therapy for FD.

Research frontiers

The major pathophysiological mechanism of PDS is gastric motility disorder, therefore, searching for a kind of effective treatment for PDS is the current research hotspot. Traditional Chinese medicine (TCM) has the advantage of treating PDS. Xiangsha Liujunzi decoction is a classical formula based on invigorating the spleen and has been used in clinical practice for hundreds of years. Gastric ultrasound is usually used to observe the gastric emptying (GE), but its value in assessing the promoting effect of Chinese herbal medicines (CHMs) on gastric motility has not yet been studied.

Innovations and breakthroughs

Although in the past two decades, a considerable number of clinical trials have proved the efficacy and safety of this decoction in the treatment of FD, there are still some disadvantages in assessing its efficacy. The authors use gastric ultrasound to observe proximal and distal GE rates. The advantages of the technique include being non-invasive, accurate, and reliable.

Applications

The authors provide strong evidence to support the use of CHM in PDS patients. This treatment will provide a new option for clinicians in treating PDS in the future.

Terminology

TCM, an independent medical system well known in China, has four ways of diagnosis that are look, listen, question and feel the pulse. It takes Yin-Yang and five elements as the theoretical basis, which holds that the human body is regarded as the unity of Qi, form and spirit. The basic principle of understanding and treating diseases for TCM is syndrome differentiation. CHMs are natural medicines that consist of plant medicine (root, stem, leaf and fruit), animal medicine (viscera, skin, bone, organ, *etc.*) and mineral medicine. The use of CHMs is conducted under the guidance of the theory of TCM.

Peer-review

This paper describes a new method for treating PDS. The authors designed a multicenter, randomized, double-blinded, placebo-controlled trial with two arms to examine the safety and efficacy of Xiangsha Liujunzi granules in the treatment of PDS patients. They assessed PDS symptoms using the change in PDSS, clinical global impression scale, TCM symptom scores, MOS 36-Item Short-Form Health Survey, and hospital anxiety and depression scale. Further, they assessed proximal and distal GE rates by gastric ultrasound, which is non-invasive, accurate, and reliable. This clinical trial study provides strong evidence to support the use of CHM in PDS patients. This treatment will provide a new option for clinicians in treating PDS in the future.

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P- Reviewer: Kim BJ, Kobayashi T **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Zhang FF

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5602-5609

DOI: 10.3748/wjg.v23.i30.5602

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

ORIGINAL ARTICLE

Observational Study

Combination of acoustic radiation force impulse imaging, serological indexes and contrast-enhanced ultrasound for diagnosis of liver lesions

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Author contributions: Sun XL and Liang LW designed the research; Sun XL, Yao H, Men Q, Hou KZ and Liang LW performed the research; Sun XL, Yao H, Men Q, Hou KZ, Chen Z, Xu CQ and Liang LW contributed new reagents or analytic tools; Sun XL, Hou KZ, Chen Z, Xu CQ and Liang LW analyzed the data; Sun XL and Liang LW wrote the paper.

Supported by Yangpu District Health and Family Planning Commission, Yangpu District Science and Technology Commission, No. YP15M18; Research Project of Shanghai Municipal Commission of Health and Family Planning, No. 201540032.

Institutional review board statement: This study was approved by Shanghai Yangpu District East Hospital Institutional Review Board.

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

Conflict-of-interest statement: No potential conflicts of interest relevant to this article are reported.

Data sharing statement: No additional data are available.

Manuscript source: Unsolicited manuscript

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Received: March 27, 2017
Peer-review started: March 29, 2017
First decision: April 11, 2017
Revised: April 24, 2017
Accepted: May 9, 2017
Article in press: May 9, 2017
Published online: August 14, 2017

Abstract

AIM

To assess the value of combined acoustic radiation force impulse (ARFI) imaging, serological indexes and contrast-enhanced ultrasound (CEUS) in distinguishing between benign and malignant liver lesions.

METHODS

Patients with liver lesions treated at our hospital were

included in this study. The lesions were divided into either a malignant tumor group or a benign tumor group according to pathological or radiological findings. ARFI quantitative detection, serological testing and CEUS quantitative detection were performed and compared. A comparative analysis of the measured indexes was performed between these groups. Receiver operating characteristic (ROC) curves were constructed to compare the diagnostic accuracy of ARFI imaging, serological indexes and CEUS, alone or in different combinations, in identifying benign and malignant liver lesions.

RESULTS

A total of 112 liver lesions in 43 patients were included, of which 78 were malignant and 34 were benign. Shear wave velocity (SWV) value, serum alpha-fetoprotein (AFP) content and enhancement rate were significantly higher in the malignant tumor group than in the benign tumor group (2.39 ± 1.20 m/s *vs* 1.50 ± 0.49 m/s, 18.02 ± 5.01 ng/mL *vs* 15.96 ± 4.33 ng/mL, 2.14 ± 0.21 dB/s *vs* 2.01 ± 0.31 dB/s; $P < 0.05$). The ROC curve analysis revealed that the areas under the curves (AUCs) of SWV value alone, AFP content alone, enhancement rate alone, SWV value + AFP content, SWV value + enhancement rate, AFP content + enhancement rate and SWV value + AFP content + enhancement rate were 85.1%, 72.1%, 74.5%, 88.3%, 90.4%, 82.0% and 92.3%, respectively. The AUC of SWV value + AFP content + enhancement rate was higher than those of SWV value + AFP content and SWV value + enhancement rate, and significantly higher than those of any single parameter or the combination of any two of parameters.

CONCLUSION

The combination of SWV, AFP and enhancement rate had better diagnostic performance in distinguishing between benign and malignant liver lesions than the use of any single parameter or the combination of any two of parameters. It is expected that this would provide a tool for the differential diagnosis of benign and malignant liver lesions.

Key words: Combined diagnosis; Liver lesions; Benign; Malignant; Differentiation

Core tip: This study investigated the diagnostic value of combined acoustic radiation force impulse (ARFI) imaging, serological indicators and contrast-enhanced ultrasound in differentiating between benign and malignant liver lesions. The results showed that the diagnostic performance of combined ARFI, alpha-fetoprotein (AFP) and contrast-enhanced ultrasound in distinguishing between benign and malignant liver lesions was higher than the use of any single parameter or the combination of any two of parameters. It is expected that this would provide a tool for the differential diagnosis of benign and malignant liver lesions.

Sun XL, Yao H, Men Q, Hou KZ, Chen Z, Xu CQ, Liang LW. Combination of acoustic radiation force impulse imaging, serological indexes and contrast-enhanced ultrasound for diagnosis of liver lesions. *World J Gastroenterol* 2017; 23(30): 5602-5609 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5602.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5602

INTRODUCTION

The gold standard for diagnosis of benign and malignant liver lesions is pathological examination. However, due to its invasive nature and the potential presence of infection, bleeding and other risks, pathological examination is only conducted in patients highly suspected of having malignant liver tumors. In addition, pathological diagnosis has many contraindications and is applicable in only a narrow range of patients. Thus, it is difficult to implement pathological diagnosis as a routine examination. For both benign and malignant liver lesions, detection of serological indicators and contrast-enhanced ultrasonography (CEUS) are routinely performed. Serological indicators alone often have a lower sensitivity and specificity than CEUS and acoustic radiation force impulse (ARFI) imaging. However, for small hepatocellular carcinoma, alpha-fetoprotein (AFP) is the most sensitive indicator[1,2]. Although CEUS is a mature examination method, its diagnostic accuracy may be affected by lesion depth, blood flow velocity and respiratory movement, which makes it not suitable for some lesions and patients[3-9]. ARFI imaging is simple and highly accepted by patients, and it can quantitatively assess the change in tissue hardness. However, it is vulnerable to biliary and intrahepatic vascular effects[10-17]. Although these three kinds of examinations have certain value in identifying benign and malignant lesions, each has its limitations. At present, the diagnostic accuracy of the combination of these three kinds of examinations in identifying benign and malignant liver lesions remains unknown, although the combined diagnosis has been widely used for other diseases. In the present study, by constructing receiver operating characteristic (ROC) curves, we compared the diagnostic accuracy of ARFI imaging, serological indexes and CEUS, alone or in different combinations, in identifying benign and malignant liver lesions, with an aim to provide a reliable tool for the differential diagnosis of benign and malignant liver lesions.

MATERIALS AND METHODS

Study subjects

A total of 43 patients with 112 liver lesions treated

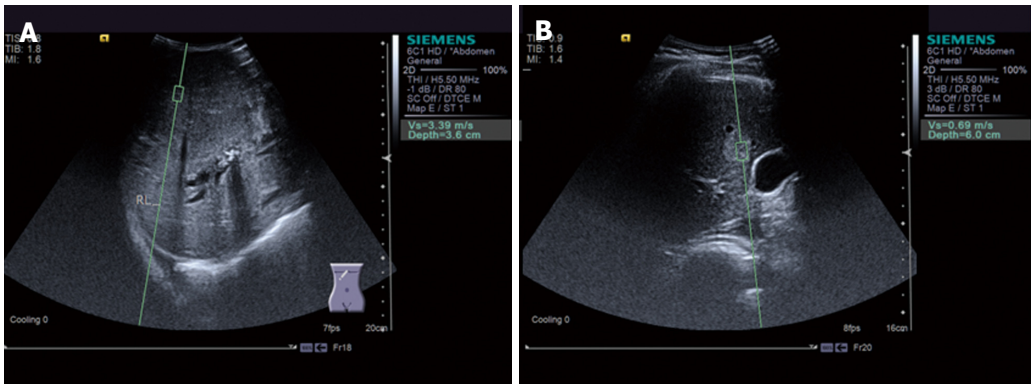

Figure 1 Acoustic radiation force impulse imaging of a liver tumor. A: Acoustic radiation force impulse (ARFI) detection of a malignant liver tumor [shear wave velocity (SWV) = 3.39 m/s]; B: ARFI detection of a benign liver tumor (SWV = 0.69 m/s).

at our hospital from May 2016 to February 2017 were included in this study. Among these patients, 21 were male and 22 were female. The lesion size ranged from 8 mm × 7 mm to 123 mm × 100 mm. The exclusion criteria were: (1) patients in whom shear wave velocity (SWV) values could not be acquired during ARFI imaging; (2) patients who could not tolerate the ultrasound contrast agent or critically ill patients; (3) patients with completely liquefied cystic tumors; and (4) patients with contraindications to liver puncture biopsy. The study was approved by the Ethics Committee of Shanghai Yangpu District East Hospital, and informed consent was obtained from all patients.

Instruments and methods

ARFI imaging: A Siemens Acuson S3000 color Doppler ultrasound system with a 6C-1HD convex array probe (center frequency, 4.0 MHz) was used. First, a whole liver scan was performed using conventional ultrasound to observe the nature of the lesion. Then, this was switched to ARFI mode to avoid biliary ducts within the liver, blood vessels and liquid necrosis. Patients were instructed to completely hold their breath during the examination. Then, SWV measurement was initiated while keeping the probe stationary. This was repeated for 3 to 6 times at the lesion center, and repeated for 3 to 5 times at the periphery of the lesion, with average SWV value calculated.

CEUS: CEUS was performed with the same system for ARFI imaging in the contrast mode, with the mechanical index adjusted to the appropriate state. SonoVue suspension (2.0 mL) was administered by bolus injection *via* the elbow vain, followed by rapid injection of 5 mL of normal saline. The patient was breathing steadily throughout the procedure. After imaging, the records were exported. Then, analysis software was use to plot the time intensity curve of the lesion and obtain the initial time, peak time, initial strength, peak intensity and 180-s echo intensity of the lesion. Subsequently, peak acceleration time, intensity increment, enhancement rate, and 180-s dissipation rate were calculated.

Serological examination: An automatic biochemical analyzer was used for the quantitative analysis of the following serological indicators: AFP, serum fucosidase (AFU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltranspeptidase (GGT), and alkaline phosphatase (ALP).

Statistical analysis

SPSS 17.0 statistical software was used for statistical analyses. Measurement data are presented as mean ± SD. Comparison of data between groups was performed using two independent samples *t*-test. Indicators with statistically significant differences were used to construct the ROC curves to calculate the area under the curve (AUC), in order to investigate the diagnostic accuracy. Diagnostic accuracy was compared between different combinations to determine the value of combined diagnosis in identifying benign and malignant liver lesions.

RESULTS

Pathological results

Pathological or radiological diagnosis confirmed 78 cases of malignant tumors and 34 cases of benign tumors. Among these cases, 18 were primary liver cancer, 60 were metastatic cancer, 16 were hemangiomas, 11 were liver cysts, 5 were adenomas, and 2 were focal nodular hyperplasia.

Comparison of SWV values between malignant and benign tumor groups

SWV values were significantly higher in the malignant tumor group (2.39 ± 1.20 m/s, a typical image is shown in Figure 1A) than in the benign tumor group (1.50 ± 0.49 m/s, a typical image is shown in Figure 1B) ($P < 0.05$; Figure 2).

Comparison of serological indicators between malignant and benign tumor groups

AFP level was significantly higher in the malignant

Table 1 Comparison of serological indicators between the malignant and benign tumor groups

	AFP (ng/mL)	AFU (U/L)	ALT (U/L)	AST (U/L)	GGT (U/L)	ALP (U/L)
Malignant tumor group	18.02 ± 5.01	29.74 ± 14.22	39.01 ± 3.60	46.92 ± 12.11	62.40 ± 27.30	139.40 ± 85.90
Benign tumor group	15.96 ± 4.33	25.61 ± 13.11	37.90 ± 4.33	44.40 ± 11.60	54.90 ± 22.30	117.99 ± 57.30
t-value	2.081	1.446	1.705	1.409	1.409	1.328
P value	0.04	0.151	0.09	0.162	0.162	0.187

AFP: Alpha-fetoprotein; AFU: Serum fucosidase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GGT: Glutamyltranspeptidase.

Table 2 Comparison of contrast-enhanced ultrasound parameters in the malignant and benign tumor groups

	Start time (s)	Initial strength (dB)	Peak time (s)	Peak intensity (dB)	120s Echo intensity (dB)	Peak acceleration time (s)	Intensity increment (dB)	Enhancement rate (dB/s)	Extinction rate (dB/s)
Malignant tumor group	11.98 ± 2.95	8.35 ± 6.03	30.22 ± 9.65	39.27 ± 6.32	27.33 ± 17.86	17.94 ± 4.64	30.99 ± 6.67	2.24 ± 0.21	0.08 ± 0.07
Benign tumor group	13.01 ± 3.92	6.57 ± 5.66	33.33 ± 11.96	37.77 ± 7.30	32.01 ± 11.96	20.10 ± 10.32	31.22 ± 7.12	2.01 ± 0.31	0.06 ± 0.06
t-value	-1.532	1.463	-1.456	1.101	-1.396	-1.533	-0.164	2.589	1.449
P value	0.128	0.146	0.148	0.273	0.166	0.128	0.870	0.011	0.150

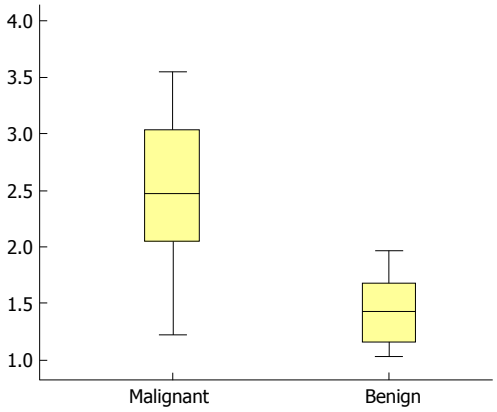

Figure 2 Shear wave velocity values of the malignant and benign tumor groups. The shear wave velocity of malignant tumors was 2.39 ± 1.20 m/s, while that of benign tumors was 1.50 ± 0.49 m/s.

tumor group than in the benign tumor group ($P < 0.05$), while the differences in AFU, ALT, AST, GGT and ALP levels were not statistically significant ($P > 0.05$) (Table 1).

Comparison of CEUS indicators between malignant and benign tumor groups

CEUS enhancement rate was significantly higher in the malignant tumor group than in the benign tumor group (a typical image is shown in Figure 3A and B) ($P < 0.05$), while the differences in start time, initial strength, peak time, peak intensity, 180-second echo intensity, peak acceleration time, strength increment, and rate of regression were not statistically significant ($P > 0.05$) (Table 2).

Diagnostic accuracy of different combinations of SWV value, AFP content and enhancement rate

SWV value, AFP content and enhancement rate were combined in the following different ways: SWV value + AFP content, SWV value + enhancement rate, AFP content + enhancement rate, and SWV value + AFP content + enhancement rate. Then, the ROC curves were constructed to calculate the AUCs. The AUCs of SWV value alone, AFP content alone, enhancement rate alone, SWV value + AFP content, SWV value + enhancement rate, AFP content + enhancement rate and SWV value + AFP content + enhancement rate were 85.1%, 72.1%, 74.5%, 88.3%, 90.4%, 82.0% and 92.3%, respectively. Taking the maximum value of the Youden index, SWV = 1.60 m/s, AFP = 18.68 ng/mL, and enhancement rate = 2.21 dB/s were determined as the best cut-off values for the diagnosis of benign and malignant liver lesions (Figures 4 and 5). When the cut-off value for the regression coefficient of the SWV value, AFP content and enhancement rate was 0.8711451, it was found that 23 cases were false-negative lesions, which included 11 cases of liver metastases derived from the digestive tract and 12 cases of hepatocellular carcinoma; and there were no false-positive lesions.

DISCUSSION

Pathological examination is the gold standard for diagnosing liver lesions. However, patient acceptance is low due to the invasiveness of the examination. Furthermore, pathological examination cannot be routinely used for screening of benign and malignant liver lesions. Serological indicators and CEUS are commonly used noninvasive examinations in clinical practice. However, serological indicators alone have a low sensitivity and specificity, and the CEUS examination process is complex and requires some skills[18-21]. ARFI imaging is simple and can quantitatively reflect changes in tissue hardness. Studies have shown that ARFI can be used to diagnose benign and malignant

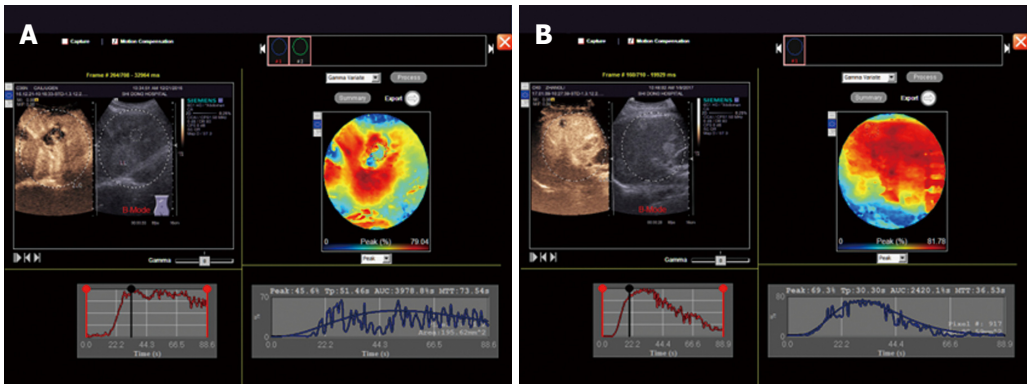

Figure 3 Contrast-enhanced ultrasound TIC curves used to detect benign and malignant liver tumors. A: TIC curve for the detection of malignant liver tumors; B: TIC curve for the detection of benign liver tumors.

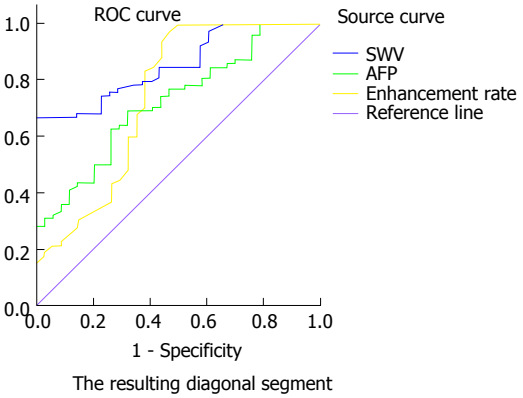

Figure 4 Comparison of the receiver operating characteristic curves of shear wave velocity, alpha-fetoprotein and enhancement rate for the identification of benign and malignant liver tumors.

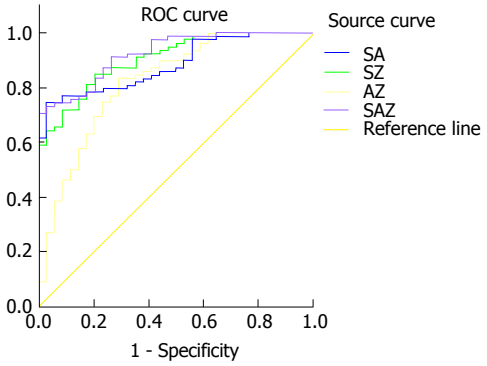

Figure 5 Comparison of the receiver operating characteristic curves of the combination of shear wave velocity value, alpha-fetoprotein content and enhancement rate for identifying benign and malignant liver tumors. SA = SWV value + AFP content; SZ = SWV value + enhancement rate; AZ = AFP content + enhancement rate; SAZ = SWV value + AFP content + enhancement rate.

liver tumors, but it is easily affected by bile ducts and large blood vessels[22-29]. Thus, each of these three types of detection methods has its own advantages and disadvantages. Since the combined diagnosis has been widely used for other diseases at present, we hypothesized that the combination of these three kinds of examinations might have better accuracy in identifying benign and malignant liver lesions. Therefore, we constructed ROC curves to compare the diagnostic efficacy of ARFI imaging, serological indexes and CEUS, alone or in different combinations, in the present study.

Comparison of ARFI, serological parameters, CEUS indicators between malignant and benign tumor groups

Our results revealed that SWV value, AFP content and CEUS enhancement rate were significantly higher in the malignant tumor group than in the benign tumor group, suggesting that the use of these parameters is feasible for the differential diagnosis of benign and malignant liver lesions. Increased SWV value may indicate increased tumor hardness. This may be due to the richness of the liver cancer substance in tumor cells that sustains growth and promotes the invasion of capillaries, and the chaotic arrangement of tissues that show invasive growth. Hence, activity in the surrounding tissue is limited, because the hardness of malignant liver tumors is greater than that of benign liver tumors. Elevated AFP content may be due to malignant liver cells, in which the AFP gene is re-expressed and AFP is released to blood. CEUS enhancement rate represents the rich blood supply of the tumor, because the number of microvessels per unit volume of malignant tumors is more than that of benign tumors, and the unit time into the malignant tumor contrast agent also increases. Hence, the enhancement rate is higher[3,4,30-42]. The difference in AFP and CEUS enhancement rate between benign and malignant liver tumors can be expected, and the difference in SWV value between the two groups suggests that ARFI can also be used for the analysis of benign and malignant liver lesions.

Diagnostic accuracy of SWV value, AFP content and enhancement rate, alone or in different combinations, in distinguishing between malignant and benign tumors

The results of this study showed that the AUC of SWV value was slightly higher than that of CEUS enhancement rate and significantly higher than that of

AFP content. This finding suggests that the diagnostic value of ARFI is higher than that of CEUS enhancement rate and AFP content and the diagnostic value of ARFI is higher than that of AFP. This might be because the change in the texture of liver lesions is more sensitive than that of AFP content. The diagnostic value of CEUS is less than that of ARFI, and the reason may be that CEUS reflects the hemodynamics of liver lesions, while ARFI reflects changes in tissue hardness. Tissue hardness reflects not only the vascular composition of tissue, but also the interstitial fiber composition of tissue, cell arrangement and other factors. Hence, compared to CEUS, ARFI is more comprehensive and intuitive.

The results of this study showed that the diagnostic accuracy of combined SWV value, AFP content and enhancement rate was slightly higher than that of SWV value plus AFP content and SWV value plus enhancement rate, and significantly higher than that of AFP content plus enhancement rate and any one of the three parameters. When the cut-off value for the regression coefficient of the SWV value, AFP content and enhancement rate was 0.8711451, the best diagnostic performance was achieved. With this cut-off value, the results of this study revealed that nine cases of hepatocellular carcinoma were false-negative lesions, which may have been transformed through liver cirrhosis to stage I liver cancer, in which the tumor composition was less obvious. Furthermore, SWV value and AFP content were low, which may be related to the partial necrosis in the tumor component. These led to the low SWV value, AFP content and enhancement rate regression coefficient, and then the false negative results were obtained. Meanwhile, studies have shown that the stiffness of hepatocellular carcinoma and cholangiocarcinoma is greater than that of liver metastases. Furthermore, metastatic carcinoma derived from the digestive tract has lower hardness, and AFP content is lower than the content of liver parenchyma. This led to low SWV value, AFP content and enhancement rate of regression coefficient, and then false negative results were obtained[43-51].

The results of this study were based on data from a small sample and did not cover all types of malignant and benign liver tumors. Therefore, the results of this study should be explained with caution, especially in clinical settings. However, the clinical pathological types of liver lesions studied in this study are common and have certain representative significance. Multi-center studies with a larger sample should be performed to verify our findings.

In summary, the diagnostic performance of combined ARFI, AFP and contrast-enhanced ultrasound in distinguishing between benign and malignant liver lesions was higher than the use of any single parameter or the combination of any two of parameters. It is expected that this would provide a tool for the differential diagnosis of benign and malignant liver lesions.

COMMENTS

Background

The gold standard for identifying benign and malignant liver lesions is pathological diagnosis. However, due to its invasiveness, the presence of infection, bleeding and other risks, pathological examination may cause body damage. Hence, pathological examination is only suitable for patients with suspected liver malignancy. In addition, pathological diagnosis has many contraindications and is applicable in only a narrow range of patients. Thus, it is difficult to implement pathological diagnosis as a routine examination.

Research frontiers

Serological indicators and contrast-enhanced ultrasound (CEUS) are noninvasive routine examinations for both benign and malignant liver lesions. Serological indicators alone have a low specificity and sensitivity. CEUS is a well-established examination method; however, it is relatively complex to operate and has high technical requirements, and some patients cannot tolerate the contrast agent. Acoustic radiation force impulse (ARFI) imaging is simple to operate, has high patient acceptance, and can quantitatively reflect the change in tissue hardness; however, it is easily affected by bile ducts and large blood vessels. The diagnostic value of the combined of ARFI imaging, serological indicators, and CEUS remains unclear.

Innovations and breakthroughs

Although ARFI imaging, serological indexes and CEUS have certain value in identifying benign and malignant lesions, each has its limitations. At present, the diagnostic accuracy of the combination of these three kinds of examinations in identifying benign and malignant liver lesions remains unknown, although the combined diagnosis has been widely used for other diseases. The results of this study showed that the diagnostic performance of combined ARFI, serum alpha-fetoprotein (AFP) and contrast-enhanced ultrasound in distinguishing between benign and malignant liver lesions was higher than the use of any single parameter or the combination of any two of parameters.

Applications

It is expected that the combination of ARFI imaging, serological indicators and CEUS would provide a tool for the differential diagnosis of benign and malignant liver lesions.

Peer-review

The combination of acoustic radiation force impulse imaging, serological indicators, and contrast-enhanced ultrasound has high accuracy in identifying benign and malignant liver lesions. This result should be confirmed by large multi-center studies, in order to better promote its clinical use.

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P- Reviewer: Hoetker MS, Meropol NJ **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Li D

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5610-5618

DOI: 10.3748/wjg.v23.i30.5610

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

ORIGINAL ARTICLE

Prospective Study

Incidents and adverse events of endoscopic ultrasound-guided fine-needle aspiration for pancreatic cystic lesions

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Author contributions: Chai NL, Linghu EQ and Li HK designed the research; Du C, Chai NL, Linghu EQ, Li HK, Sun YF, Xu W, Wang XD, Tang P and Yang J performed the research; Du C analyzed the data; and Du C wrote the paper.

Institutional review board statement: The clinical trial described in this paper was reviewed and approved by the Committee of Medical Ethics of Chinese PLA General Hospital.

Clinical trial registration statement: The study is registered at http://www.chictr.org.cn/showproj.aspx?proj=10604. The registration identification number is ChiCTR-OOC-15006118.

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

Conflict-of-interest statement: There are no conflicts of interest in relation to this manuscript.

Data sharing statement: There are no additional data available in relation to this manuscript.

Manuscript source: Unsolicited manuscript

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Received: April 15, 2017
Peer-review started: April 15, 2017
First decision: June 1, 2017
Revised: June 12, 2017
Accepted: June 18, 2017
Article in press: June 19, 2017
Published online: August 14, 2017

Abstract

AIM

To evaluate the diagnostic value and safety mainly regarding incidents of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) for pancreatic cystic lesions (PCLs).

METHODS

A total of 150 consecutive patients with suspected PCLs were prospectively enrolled from April 2015 to November 2016. We finally enrolled 140 patients undergoing EUS-FNA. We compared the diagnostic accuracy of EUS-FNA and pathological diagnosis, which is regarded as the gold standard, for PCLs. Patients undergoing EUS-FNA at least 1 wk preoperatively were monitored for incidents and adverse events to evaluate its safety.

RESULTS

There were 88 (62.9%) women and 52 (37.1%) men among 140 patients, with a mean age of 50.1 (± 15.4) years. There were 67 cysts located in the head/

uncinate of the pancreas and 67 in the body/tail, and 6 patients had at least 1 cyst in the pancreas. There were 75 patients undergoing surgery and 55 undergoing EUS-FNA with interval at least 1 wk before other operations, with 3 patients undergoing the procedure twice. The accuracy of EUS-FNA in differentiating benign and malignant lesions was 97.3% (73/75), while the accuracy of characterizing PCL subtype was 84.0% (63/75). The incident rate was 37.9% (22/58), whereas only 1 AE was observed in 58 cases.

CONCLUSION

EUS-FNA is effective and safe for diagnosis of PCLs, however procedure-related incidents are common. Caution should be taken in patients undergoing EUS-FNA.

Key words: Endoscopic ultrasound; Incident; Fine-needle aspiration; Pancreatic cystic lesion

Core tip: Incidents are self-limiting and do not change therapy. Adverse events (AEs) of endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) have attracted attention, whereas incidents are almost ignored. Although incidents do not interfere with procedures and treatment, documenting them might improve procedural quality and prediction of AEs. Our study was designed to evaluate the diagnostic value and safety mainly regarding incidents of EUS-FNA. We found the accuracy of EUS-FNA in differentiating benign and malignant lesions and characterizing pancreatic cystic lesions subtype was high. The AE rate was low, however procedure-related incidents are common and should be paid attention to.

Du C, Chai NL, Linghu EQ, Li HK, Sun YF, Xu W, Wang XD, Tang P, Yang J. Incidents and adverse events of endoscopic ultrasound-guided fine-needle aspiration for pancreatic cystic lesions. *World J Gastroenterol* 2017; 23(30): 5610-5618 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5610.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5610

INTRODUCTION

Pancreatic cystic lesions (PCLs) are becoming increasingly prevalent, with increased diagnosis related to the wide use of abdominal cross-sectional imaging. The incidence of asymptomatic cysts ranges from 0.7% to 24.3%[1-5]. With a broad differential diagnosis, PCLs are mainly divided into benign non-neoplastic cysts and neoplastic cysts, some of which have malignant potential or are of low malignancy. The frequency of malignancy among mucinous cystic neoplasms (MCNs) and intraductal papilla mucinous neoplasms (IPMNs) which are subtypes of neoplastic cysts, ranges from 3.9% to 81%[6], while the 2-year survival rate of malignant PCLs is as low as 10%[7].

Correct diagnosis and accurate classification of PCLs are important for making treatment decision. Endoscopic ultrasound (EUS) has high spatial resolution, and EUS-guided fine-needle aspiration (EUS-FNA) contributes to diagnosis by providing cystic fluid examination, cytology and biopsy[8]. EUS-FNA is the predominant method for diagnosis of PCLs[9,10]. However, compared with computed tomography (CT) and magnetic resonance imaging (MRI), EUS-FNA is an invasive operation. It is the top priority to ensure the safety of EUS-FNA.

Incidents are unplanned events that have no influence on completion of an operation and post-operative treatment, and adverse events (AEs) are defined as events that prevent completion of or change to the planned procedure[11]. Incidents are self-limiting and do not change therapy. AEs have attracted attention, whereas incidents are almost ignored. Although incidents do not interfere with procedures and treatment, documenting them might improve procedural quality and predict AEs.

There have been many studies on the safety and diagnostic accuracy of EUS-FNA for PCLs, but there have been few studies regarding the incidents related to this procedure. Our study was designed to evaluate the diagnostic value and safety mainly regarding incidents of EUS-FNA.

MATERIALS AND METHODS

Patients

We prospectively enrolled 150 consecutive patients with suspected PCLs from April 2015 to November 2016. Excluding 10 patients who did not undergo EUS-FNA, we finally enrolled 140 patients. The indications for EUS-FNA were: (1) indeterminate PCLs in radiological imaging studies; (2) easier and safer access to the cyst; (3) age $\geqslant$ 18 years; and (4) signed informed consent. The following exclusion criteria were used: (1) reluctance to receive EUS-FNA or inability to sign informed consent independently; (2) high risk for operation, or pregnancy; (3) evidence of active acute pancreatitis, pancreatic necrosis or pseudocyst; and (4) coagulopathy (international normalized ratio > 1.5, platelets < 50000). When evaluating the diagnostic value, only the patients who underwent surgery were enrolled. When evaluating the safety of EUS-FNA, patients who did not undergo any other operation > 1 wk after EUS-FNA were studied.

Study design

Patients with suspected PCLs by imaging examination were requested to undergo EUS examination and EUS-FNA. The EUS and EUS-FNA procedures were performed by experts with > 10 years' experience. Some patients underwent other operations, like surgery,

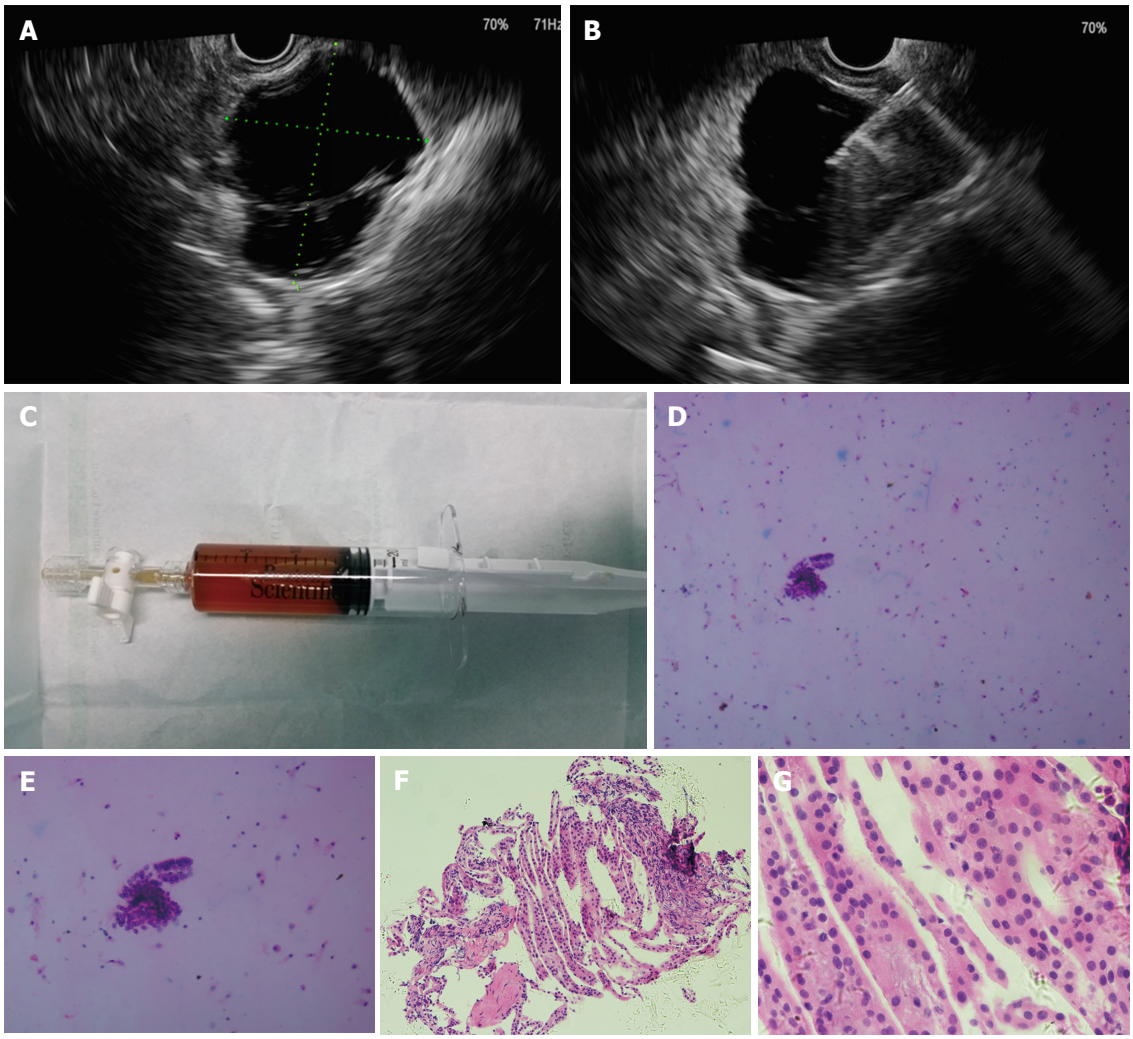

Figure 1 The procedures of endoscopic ultrasound-guided fine-needle aspiration. A: Endoscopic ultrasound view of the cyst, showing a 46.0 mm^2 × 39.0 mm^2 cyst in pancreatic neck; B: Puncture of the cyst with a 19-guage needle and aspiration of the cystic fluid; C: Specimen of cystic fluid, sent for cytology and biochemical analysis; D: Histopathological image of cystic fluid cytology, diagnosed with serous cystic neoplasm (H and E, × 100); E: Histopathological image of cystic fluid cytology (H and E, × 200); F: Histopathological image of biopsy of the cystic wall of the same cyst (H and E, × 10); G: Histopathological image of biopsy of the cystic wall of the same cyst (H and E, × 40).

EUS-guided ablation and endoscopic retrograde cholangiopancreatography, after the EUS-FNA. The presumed endoscopic diagnosis was made after taking EUS and cystic fluid examination findings into consideration. The diagnostic accuracy of EUS-FNA was compared with pathological diagnosis, which is regarded as the gold standard for diagnosis of PCL.

Patients undergoing EUS-FNA ⩾ 1 wk before other operations were monitored for incidents and AEs to evaluate safety; therefore, patients who underwent other operations < 1 wk after EUS-FNA were excluded when evaluating the incident and AE rates. Any symptoms and signs of abdominal pain, fever, bleeding, nausea, infection, acute pancreatitis, perforation and hyperamylasemia, were recorded. Patients were monitored on the ward for ⩾ 3 d and discharged when they did not feel any discomfort. If they were hospitalized for < 7 d, we followed them up by telephone to document incidents and AEs that might have arisen.

Endoscopic procedures

All patients with suspected PCLs underwent EUS evaluation with a liner-array echoendoscope (Prosound F75; Aloka, Tokyo, Japan, and GF-UCT260; Olympus, Tokyo, Japan) under intravenous anesthesia. The lesions were characterized by size, location, wall thickness, number of septa, morphology of the pancreatic duct, and presence of papilla or associated mass. Transgastric or transduodenal puncture of the cyst was done using a 22-gauge or 19-gauge needle (Echotip; Cook, Limerick, Ireland) and cystic fluid was aspirated. If the cystic fluid was too viscous for aspiration, 0.9% normal saline solution was used to decrease the viscosity of the cyst. The cyst fluid was sent for cytology and biochemical analysis. Biopsy of the cystic wall through a fine needle was done if necessary. The procedures are shown in Figure 1.

Table 1 Baseline characteristics of 140 patients suspected of pancreatic cystic lesions

Characteristic	Result[1]
Age, yr	50.1 ± 15.4
Sex	
Female	88 (62.9)
Male	52 (37.1)
Cyst location	
Head/uncinate	67 (47.9)
Body/tail	67 (47.8)
Multiple cysts	6 (4.3)
Pathological diagnosis	
Neoplastic cyst	70
MCN	25
SCN	27
SPN	7
IPMN	8
NEN	1
Cystadenocarcinoma	2
Non-neoplastic cyst	5
Pseudo cyst	2
True cyst	2
Cystic tuberculosis	1

[1]Presented as mean ± SD, *n* (%), or *n*. IPMN: Intraductal papilla mucinous neoplasm; MCN: Mucinous cystic neoplasm; SCN: Serous cystic neoplasm; SPN: Solid pancreatic neoplasms; NEN: Neuroendocrine neoplasm.

Postoperative treatment

After EUS-FNA, patients were intravenously administered one dose of an intravenous antibiotic for 3 d and octreotide for 1 d. An intravenous proton pump inhibitor (PPI) for 1 d and an additional 3 d of an oral PPI were required. Six hours and the morning after the procedure, the patients were assessed for serum amylase and lipase levels. If these results were abnormal, rechecking was required once daily before they returned to normal. Oral intake of food was allowed 1 d after EUS-FNA if there was no severe AE.

Definitions

Incidents were different from AEs. Incidents were defined as symptoms or signs that did not interfere with the planned treatment. AEs were defined as events that prevented completion of or change to the planned procedure. Moderate to severe abdominal pain that needed additional treatment was regarded as an AE, while mild abdominal pain was regarded as an incident. The size of PCLs was determined by their largest diameter. If EUS-FNA was performed on several cysts in one patient, the diameter was calculated as the sum of the largest diameters of these cysts. All of the patients were given a presumed diagnosis on the basis of EUS, cystic fluid analysis and cystic wall biopsy before surgery.

Statistical analysis

All calculations were performed using SPSS version 17.0. Quantitative data, including cystic size and patients' age, were expressed by the mean or median and tested by *t*-test or nonparametric test. Enumeration data, like diagnostic accuracy rate and incident rate, were tested using χ^2 or Fisher's exact test. A P value < 0.05 was considered significant.

RESULTS

Basic characteristics are summarized in Table 1. There were 88 (62.9%) women and 52 (37.1%) men among 140 patients, with a mean age of 50.1 (± 15.4) years. There were 67 cysts located in the head/uncinate of the pancreas and 67 in the body/tail, and 6 patients had at least one cyst in the pancreas. Cystic fluid analysis was available for 89 patients and 1 patient had aspiration of two cysts. The levels of carcinoembryonic antigen, amylase, lipase and carbohydrate antigen 19-9 were 4.89 ng/mL (range: 0.20-19 636.5 ng/mL), 316.75 U/L (range: 1.2-275 020 U/L), 1713.60 U/L (range: 4.4-1 594 160 U/L) and 640.75 ng/mL (range: 1.07- > 20000 ng/mL), respectively. There were 75 patients undergoing surgery and 55 undergoing EUS-FNA with interval at least 1 wk before other operations, with 3 patients undergoing the procedure twice. Seventy pancreatic neoplastic cysts and five non-neoplastic cysts were found in pathological diagnosis. There were 25 MCNs, 27 serous cystic neoplasms, 7 solid pancreatic neoplasms, 8 IPMNs, 1 neuroendocrine neoplasm and 2 cystadenocarcinomas among the neoplastic cysts, and 2 pseudocysts, 2 true cysts and 1 case of cystic tuberculosis among the non-neoplastic cysts. There were 75 patients undergoing surgery after EUS-FNA and 58 were available for safety evaluation. The study flowchart is shown in Figure 2.

Accuracy of EUS-FNA

A total of 75 patients underwent surgery after EUS and pathological diagnosis was regarded as the gold standard. There were two malignant cysts by pathology and one was misdiagnosed by EUS-FNA. The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of EUS-FNA in differentiating benign and malignant lesions were 98.6% (72/73), 50.0% (1/2), 98.6% (72/73), 50% (1/2) and 97.3% (73/75), respectively. When evaluating the capacity of characterizing subtype of PCLs, the accuracy of EUS-FNA was 84.0% (63/75).

Safety of EUS-FNA

Fifty-eight patients were available for safety evaluation and monitored for ⩾ 7 d. Only 1 patient with moderate abdominal pain received additional treatment with anisodamine and the pain was relieved. No other AE occurred, which resulted in an AE rate of 1.7%.

Incidents were reported in 22 patients, with a rate of 37.9% (22/58). Seven patients developed abdominal pain; nine hyperamylasemia; four both abdominal pain and hyperamylasemia; one abdominal pain, low-grade fever and hyperamylasemia simultaneously; and one low-grade fever and hyperamylasemia simultaneously (Table 2).

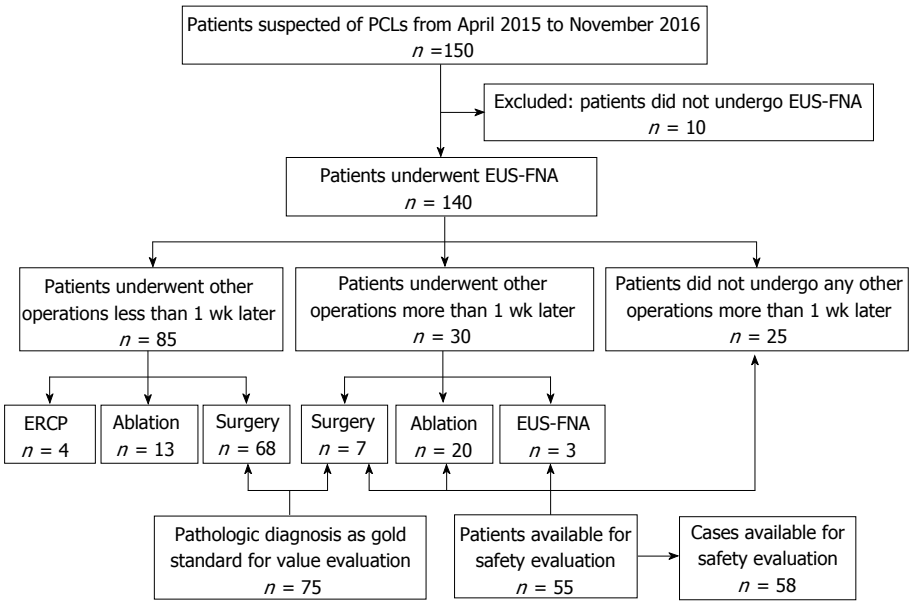

Figure 2 Study flowchart. ERCP: Endoscopic retrograde cholangiopancreatography; EUS-FNA: Endoscopic ultrasound-guided fine-needle aspiration; PCLs: Pancreatic cystic lesions.

Comparison between incidents/AEs and non-complaints of EUS-FNA

The characteristics of the incidents/AEs and non-complaints groups are described in Table 3. We performed univariate analysis of the baseline patient and cystic characteristics to predict safety related to EUS-FNA. Among the variables, no significance was shown for age, sex, location and size of the lesions.

DISCUSSION

PCLs are composed of true cysts, pseudocysts and cystic neoplasms. About 60% of PCLs are cystic tumors, followed by inflammation and trauma-related pseudocysts accounting for 30%[12]. PCLs have a wide range of lesions ranging from benign to malignant[13]. Although imaging modalities have made great advances, the accurate diagnosis of PCLs and differentiation of PCL subtypes remain challenging[13]. EUS and EUS-FNA contribute much to the diagnosis of PCLs because of their high resolution and the aid of cystic fluid and cytological analysis[14-18]. EUS-FNA can offer incremental diagnostic sensitivity with its ability to obtain cystic fluid and cytology from worrisome areas[19]. The American Gastroenterological Association Institute suggests that EUS-FNA should be used to examine PCLs with at least two high-risk features[20]. EUS-FNA might affect the management of 72% of incidental pancreatic cysts[21]. When referring to EUS-FNA, its diagnostic value and safety are the most important features for evaluating its feasibility.

Many studies have shown that the accuracy of EUS-FNA in diagnosis of PCLs ranged from 66.7% to 97%[22-25]. Under EUS-FNA, cystic fluid and cystic tissue can be collected for biochemical, cytological, genetic and pathological examination, which may help to diagnose and classify PCLs[26-28]. The diagnostic yield from combined EUS-FNA imaging is better than from EUS alone[29]. EUS-FNA contributes much to differentiation between benign and malignant PCLs[30] and between mucinous and non-mucinous cystic lesions[31,32]. Cytologic diagnosis with EUS-FNA is helpful to arrive at a more definitive diagnosis[5]. EUS with or without FNA is superior to CT and MRI in accurately classifying a cyst as neoplastic[33].

In our current study, EUS-FNA had a high sensitivity for differentiation of malignant cystic carcinoma from benign or malignant potential PCLs, but its specificity was only 50%. There were two cystic carcinomas diagnosed by EUS-FNA in our study and one was misdiagnosed. When EUS-FNA reveals malignance, we should accept its diagnosis with caution. Additional information, like age, clinical symptoms, history of present illness, blood test results and other imaging examinations, should be taken into account. However, pancreatic cancer has high malignancy with short survival time, we would rather misdiagnose than miss it. It is important to differentiate between mucinous and non-mucinous PCLs because their treatments are different.

In our research, EUS-FNA did well in classifying PCLs into different subtypes, with an accuracy of 84.0%. An earlier study suggested that diagnostic accuracy in distinguishing mucinous and non-mucinous PCLs increased up to 90% when taking cystic fluid tumor marker level, amylase level, mucin staining and cytology into consideration to make a presumed diagnosis[31]. Our result seemed lower, which may be because previous studies just made a distinction between mucinous and non-mucinous PCLs. The cystic wall puncture might increase the sensitivity of EUS-FNA[5]. A systematic review showed k-ras mutational

Table 2 Incidents of patients after endoscopic ultrasound-guided fine-needle aspiration

Incident	*n*
Abdominal pain	7
Hyperamylasemia	9
Abdominal pain + hyperamylasemia	4
Abdominal pain + low-grade fever + hyperamylasemia	1
Low-grade fever + hyperamylasemia	1

analysis used as an individual screening test has a poor diagnostic accuracy and the combined test of cytology and k-ras benefited the diagnostic value[34].

When evaluating the safety of EUS-FNA, AEs have attracted a lot of attention, with AE rates ranging from 1.14% to 14%[35-39]. A large prospective multicenter study reported a complication rate of 6%[40]. In our study, the AE rate was 1.7%. A study enrolled 414 patients showed the AEs all occurred during the first day[41]. In accordance with a previous study, pancreatitis, infection, perforation, tumor seeding and clinically significant bleeding are the most common AEs of EUS-FNA[42]. The incidence of acute pancreatitis varies from 0% to 2.6% and bacteremia can be observed in $\leqslant$ 6% of EUS procedures and EUS-FNA[18,38,40,43,44]. The incidence of abdominal pain is 0%-3.6%, while fever is reported in 0%-4.1% of cases[41,43,45-47]. No protective effect was observed from periprocedural prophylactic antibiotic administration[48]. Debate remains about whether the complications of EUS-FNA for PCLs are more frequent than for pancreatic solid lesions[39,49].

The incident rate in our study was higher than in a previous study reporting three incidents among 73 patients with PCLs and 73 with solid lesions[39]. However, the AE rate in our study was lower compared with 5.5% (4/73) in PCLs of the previous study. There are several reasons for this difference. Although the definitions of incidents and AEs are both based on an American Society for Gastrointestinal Endoscopy (ASGE) workshop, the postoperative treatment may differ. The definitions are related to planned therapy so differences in therapy will affect the discrimination between AEs and incidents. There are no clear guidelines for post-EUS-FNA treatment. Serum amylase and lipase levels were detected only when patients complained of abdominal pain in the previous study. Hyperamylasemia alone was common in our study and not necessarily accompanied by abdominal pain. Therefore, the number of incidents might have been underestimated in the previous study. Although incidents have no effect on completion of the planned procedure, paying attention to them may help optimize our treatment. For example, hyperamylasemia was reported 6 h after EUS-FNA and amylase level returned to normal the morning after the procedure. Therefore, one dose of octreotide might be enough for most patients. Noticing incidents can help operators take more care before, during and after an operation. Incidents may predict AEs, and giving attention to incidents might decrease AEs.

Table 3 Comparison between incidents/adverse events and non-complaints of endoscopic ultrasound-guided fine-needle aspiration

	Incidents/AEs group, *n* = 23	Non-complaints group, *n* = 35	*P* value
Age, yr	52.6 ± 19.0	52.6 ± 13.5	> 0.05 (NS)
Sex			> 0.05 (NS)
Female	12	18	
Male	11	17	
Location			> 0.05 (NS)
Head/uncinate	15	18	
Body/tail	5	15	
Multiple cysts	3	2	
Size by EUS mm	34.5 ± 20.1	41.2 ± 21.5	> 0.05 (NS)

Data are presented as mean ± SD or *n*. AEs: Adverse events; EUS: Endoscopic ultrasound; NS: Not significant.

To predict the incidents/AEs, we carried out univariate analysis to identify factors that might affect incidents/AEs. Incidents/AEs were similar in patients of different age and sex and with lesions of different location and size. A previous study also demonstrated that location cannot predict AEs[38]. Eloubeidi *et al*[50] reported that the type and size of the pancreatic lesion affected AEs. We speculated that factors predicting incidents and AEs were similar. However, factors that may predict incidents deserve further investigation.

Our study prospectively revealed incidents related to EUS-FNA that may help to reduce AEs. However, there were several limitations. First, although there were 140 patients enrolled, we evaluated the accuracy of EUS-FNA in 75 patients (group 1) and safety of EUS-FNA in 55 patients (group 2). They were different groups and 17 patients among enrolled patients were neither in group 1 nor in group 2. Second, although the ASGE workshop defines incidents and AEs, there is no clear guideline for post-EUS-FNA treatment. The discrimination of incidents and AEs may vary with planned treatment. The incidents in our study may be different when changes are made to postoperative treatment. The final limitation was our small number of participants. Nearly half of the EUS-FNA procedures were done followed by surgery immediately, which made the sample for safety evaluation small.

In conclusion, EUS-FNA is effective and safe for diagnosis of PCLs, and has a high diagnostic accuracy and low AE rate. However, incidents related to EUS-FNA are common. Caution should be taken in patients undergoing EUS-FNA to prevent incidents from evolving into AEs. Incidents are similar in patients of different ages and sex and with lesions of different location and size.

COMMENTS

Background

Endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) is the predominant method for diagnosis of pancreatic cystic lesions (PCLs).

Compared with computed tomography and magnetic resonance imaging, EUS-FNA is an invasive operation. It is the top priority to ensure the safety of EUS-FNA.

Research frontiers

There have been many studies on the safety and diagnostic accuracy of EUS-FNA for PCLs, but there have been few studies regarding the incidents related to this procedure. Their study was designed to evaluate the diagnostic value and safety mainly regarding incidents of EUS-FNA.

Innovations and breakthroughs

The current study noted the incidents related to EUS-FNA, which have often been ignored. EUS-FNA is safe with low incidence of adverse events (AEs). However, incidents related to EUS-FNA are common. This study also analyzed the factors that predict safety related to EUS-FNA.

Applications

Noticing incidents can help operators take more care before, during and after an operation. Incidents may predict AEs, and giving attention to incidents might decrease AEs.

Terminology

Incidents were different from AEs. Incidents were defined as symptoms or signs that did not interfere with the planned treatment. AEs were defined as events that prevented completion of or change in the planned procedure.

Peer-review

This manuscript describes an interesting investigation about the incidents and AEs of EUS-FNA for PCLs. In this study, the authors evaluated the diagnostic value and safety mainly regarding incidents of EUS-FNA for PCLs.

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P- Reviewer: Casper S, Sunanda K **S- Editor**: Wang JL
L- Editor: Filipodia **E- Editor**: Li D

World Journal of Gastroenterology

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World J Gastroenterol 2017 August 14; 23(30): 5619-5633

DOI: 10.3748/wjg.v23.i30.5619

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

SYSTEMATIC REVIEWS

Systematic review of giant gastric lipomas reported since 1980 and report of two new cases in a review of 117110 esophagogastroduodenoscopies

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Conflict-of-interest statement: None for all authors. In particular, Dr. Cappell, as a consultant of the United States Food and Drug Administration (FDA) Advisory Committee for Gastrointestinal Drugs, affirms that this paper does not discuss any proprietary confidential pharmaceutical data submitted to the FDA. Dr. Cappell is also a member of the speaker's bureau for AstraZeneca and Daiichi Sankyo, co-marketers of Movantik. This work does not discuss any drug manufactured or marketed by AstraZeneca or Daiichi Sankyo.

Data sharing statement: Consent was not obtained but the presented data are anonymized and the risk of identification is low.

Manuscript source: Invited manuscript

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Received: April 6, 2017
Peer-review started: April 11, 2017
First decision: April 17, 2017
Revised: May 4, 2017
Accepted: June 18, 2017
Article in press: June 19, 2017
Published online: August 14, 2017

Abstract

AIM

To systematically review the syndrome of giant gastric lipomas, report 2 new illustrative cases.

METHODS

Literature systematically reviewed using PubMed for publications since 1980 with following medical subject heading/keywords: ("giant lipoma") AND ("gastric") OR [("lipoma") and ("gastric") and ("bleeding")]. Two authors independently reviewed literature, and decided by consensus which articles to incorporate. Computerized review of pathology/endoscopy records at William Beaumont Hospitals, Royal Oak and Troy, Michigan, January 2005-December 2015, revealed

2 giant gastric lipomas among 117110 consecutive esophagogastroduodenoscopies (EGDs), which were thoroughly reviewed, including re-review of original endoscopic photographs, radiologic images, and pathologic slides.

RESULTS

Giant gastric lipomas are extremely rare: 32 cases reported since 1980, and 2 diagnosed among 117110 consecutive EGDs. Average patient age = 54.5 ± 17.0 years old (males = 22, females = 10). Maximal lipoma dimension averaged 7.9 cm ± 4.1 cm. Ulcerated mass occurred in 21 patients. Lipoma locations: antrum-17, body-and-antrum-4, antrum-intussuscepting-into-small-intestine-3, body-2, fundus-1, and unspecified-5. Intramural locations included submucosal-22, subserosal-2, and unspecified-8. Presentations included: acute upper gastrointestinal (UGI) bleeding-19, abdominal pain-5, nausea/vomiting-5, and asymptomatic-3. Symptoms among patients with UGI bleeding included: weakness/fatigue-6, abdominal pain-4, nausea/vomiting-4, early-satiety-3, dizziness-2, and other-1. Their hemoglobin on admission averaged 7.5 g/dL ± 2.8 g/dL. Patients with GI bleeding had significantly more frequently ulcers than other patients. EGD was extremely helpful diagnostically (n = 31 patients), based on characteristic endoscopic findings, including yellowish hue, well-demarcated margins, smooth overlying mucosa, and endoscopic cushion, tenting, or naked-fat signs. However, endoscopic mucosal biopsies were mostly non-diagnostic (11 of 12 non-diagnostic). Twenty (95%) of 21 abdominal CTs demonstrated characteristic findings of lipomas, including: well-circumscribed, submucosal, and homogeneous mass with attenuation of fat. Endoscopic-ultrasound showed characteristic findings in 4 (80%) of 5 cases: hyperechoic, well-localized, mass in gastric-wall-layer-3. Transabdominal ultrasound and UGI series were generally less helpful. All 32 patients underwent successful therapy without major complications or mortality, including: laparotomy and full-thickness gastric wall resection of tumor using various surgical reconstructions-26; laparotomy-and-enucleation-2; laparoscopic-transgastric-resection-2; endoscopic-mucosal-resection-1, and other-1. Two new illustrative patients are reported who presented with severe UGI bleeding from giant, ulcerated, gastric lipomas.

CONCLUSION

This systematic review may help standardize the endoscopic and radiologic evaluation and therapy of patients with this syndrome.

Key words: Esophagogastroduodenoscopy Lipoma; Gastric; Giant; Melena; Upper gastrointestinal bleeding; Systematic review

Core tip: Systematic literature review of giant gastric lipomas revealed 32 reported cases since 1980, with 2 new cases reported among 117110 esophagogastroduodenoscopies. Two authors independently reviewed literature, and decided by consensus which articles to incorporate. Average-patient-age = 54.5 ± 17.0 years (males = 68.8%). Mean-maximal-lipoma-diameter = 7.9 cm ± 4.1 cm. Lipoma locations: antrum-17, antrum and other gastric segments-7, other-8. Lipomas were submucosal-92%, subserosal-8%. Presentations included: acute upper gastrointestinal (UGI) bleeding-19, abdominal pain-5, nausea/vomiting-5, asymptomatic-3. Esophagogastroduodenoscopy was extremely helpful diagnostically; findings included: yellowish hue, well-demarcated margins, and smooth overlying mucosa. Endoscopic biopsies were infrequently diagnostic. Twenty of 21 abdominal CTs demonstrated characteristic lipoma findings: well-circumscribed, submucosal, and homogeneous mass with fat attenuation. Endoscopic-ultrasound showed characteristic findings in 80%. All patients underwent successful therapy without major complications/mortality, including: laparotomy-with-full-thickness-gastric-wall-resections-26; and other-6. Two newly reported patients presented with severe UGI bleeding from giant, ulcerated, gastric lipomas. This review may help standardize work-up of these patients.

Cappell MS, Stevens CE, Amin M. Systematic review of giant gastric lipomas reported since 1980 and report of two new cases in a review of 117110 esophagogastroduodenoscopies. *World J Gastroenterol* 2017; 23(30): 5619-5633 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5619.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5619

INTRODUCTION

Gastric lipomas are rare, constituting < 3% of benign gastric tumors, and < 1% of all gastric tumors[1], and giant gastric lipomas (⩾ 4 cm) are extremely rare, with only 32 cases reported since 1980 (Table 1)[1-33]. Although small gastric lipomas are usually asymptomatic, giant gastric lipomas typically produce major symptoms from GI obstruction, tumor ulcers, or acute upper gastrointestinal (UGI) bleeding, with 19 cases of UGI bleeding reported since 1980 (Table 1). Due to its extreme rarity, all prior studies of giant gastric lipomas have comprised single case reports. This work systematically reviews the literature since 1980, and collates the case reports scattered among various and sometimes obscure journals, to semi-quantitatively describe the clinical presentation, endoscopic and radiologic findings, and therapy of the disease; and to report two new illustrative cases who presented with massive, life-threatening UGI bleeding among 117110

Table 1 Comprehensive review of the 32 giant gastric lipomas reported since 1980

Ref.	Age, sex, clinical presentation, PMH, signs and lab abnormalities	Diagnostic work-up	Treatment, pathology	Outcome and follow-up
Upper GI bleeding				
Current case report 1	63 y. o. M with previous medical history of hypertension and hyperlipidemia presented with melena and dyspnea on exertion for 3 d and epigastric pain, early satiety and 10-kg weight loss during the last 6 mo. BP = 144/77 mm/Hg, pulse = 87/min. Hgb = 6.2 g/dL	EGD: 13-cm-wide, submucosal, yellowish, gastric mass in antrum covered by smooth mucosa except for focal ulceration Abdominal CT: well-circumscribed, uniform 13.4 cm × 8.4 cm × 8.2 cm mass, with attenuation characteristic for fat	Laparotomy: Resected by subtotal gastrectomy extended by partial bulbar duodenectomy with Billroth Ⅱ reconstruction Pathology: Homogeneous, submucosal, soft, 14.5 cm × 8.7 cm × 7.5 cm mass. Lipoma with spindle cell variant by CD34 positivity by immunohistochemistry	Did well postoperatively with no complications. Asymptomatic at 8 wk of follow-up
Current case report 2	78 y. o. F presented with melena for 3 d, associated with weakness and orthostatic dizziness. BP = 124/67 mmHg, pulse = 68/min. Rectal exam-melena. Hgb = 7.1 g/dL	Abdominal CT: submucosal, 9.5 cm × 6.0 cm × 4.5 cm, antral mass. EGD: large, focally ulcerated, antral gastric mass, exhibiting a positive cushion sign	Laparotomy: large, 9.0 cm × 6.0 cm × 4.5 cm, submucosal mass excised by distal gastrectomy. Pathology: lipoma	Patient discharged 5 d postoperatively with no further bleeding
Ramdass *et al*[1], 2013	37 y. o. F with epigastric pain, melena, vomiting and weakness for 4 d. Pallor and epigastric tenderness. Hgb = 5.9 g/dL. Transfused 6 units packed erythrocytes	EGD: submucosal mass with 1 cm central ulcer in gastric body	Gastric body. Laparotomy: 4 cm × 3.5 cm × 3.2 cm mass at junction of body and antrum removed surgically Pathology: lipoma	Did well postoperatively with uneventful recovery
Almohsin *et al*[2], 2015	61 y. o. M presented with hematemesis, melena, epigastric pain, and fatigue	EGD: Gastric mass with an ulcer. Endoscopic biopsies: benign tissue. EUS: large, hyperechoic, antral, submucosal lesion. Abdominal CT: 8.5 cm × 5 cm submucosal, well-encapsulated antral lesion with density of fat with ulcerated overlying mucosa	Laparotomy: enucleation of lesion and overlying mucosa. Pathology: lipoma	Remained well at 9 mo follow-up
Beck *et al*[3],1997	13 y, o. M with hematemesis, melena and abdominal pain for 2 d. Occasional nausea and vomiting for several years. Benign abdomen Hgb = 10.5 g/dL	Abdominal radiograph: polypoid mass. EGD: 8 cm × 3 cm × 4 cm soft and compressible, polypoid mass with basal ulceration on anterolateral wall of antrum. Endoscopic mucosal biopsy: normal antral tissue. Abdominal CT: smooth, uniform intraluminal mass with low attenuation in submucosal layer	Endoscopic polypectomy: Unsuccessful due to thick polyp stalk and patient pain during attempted polypectomy Surgery: Excision of polyp Pathology: lipoma	Uneventful postoperative course. Patient asymptomatic
Bijlani *et al*[4], 1993	70 y. o. M presented with acute hematemesis. Physical examination revealed pallor. Hgb = 7.0 g/dL	EGD: Protruding mass in antrum. Could not traverse endoscope beyond mass. Endoscopic biopsies: normal UGI series: space-occupying lesion in antrum Abdominal USD: normal	Laparotomy: soft, yellowish mass in antrum stretching the serosa. Mass enucleated *via* serosal approach Pathology: lipoma	Uneventful post-operative recovery. Asymptomatic at 6 mo of follow-up
Bloch *et al*[5], 1974	55 y, o. F with 1 episode of melena Nausea, epigastric fullness, and belching for 7 mo. Physical exam reveals grapefruit-sized epigastric mass N.A	Supine abdominal radiograph: Well-demarcated, large epigastric mass UGI series: huge, sharply demarcated, mass in distal two-thirds of stomach with 2 cm × 3 cm ulcer at apex of mass	Distal two-thirds of stomach on anterior wall. Laparotomy: huge, grapefruit size submucosal lipoma arising from anterior wall with shallow central ulcer Surgical resection: not documented	N.A
Chu *et al*[6], 1983	61 y. o. F with previous medical history of gastric ulcer and hiatal hernia diagnosed 2 yr earlier presented with melena and weakness for several days. Rectal exam: fecal occult blood. Hgb = 6.0 g/dL. Transfused 3 units of packed erythrocytes	UGI series: sliding hiatal hernia, and golf-ball-sized mass protruding from lesser curve in antrum. Mass moved in and out of pylorus EGD: well-circumscribed, submucosal, 5 cm × 3 cm-mass protruding along lesser curve in antrum. Positive cushion sign	Laparotomy: 5 cm × 4 cm × 3 cm mass in pre-pylorus. Underwent resection of mass with adjacent lesser curvature, and pyloroplasty Pathology: lipoma	Uneventful postoperative course and asymptomatic at 1 yr
Kibria *et al*[7], 2009	44 y. o. F with hematemesis and melena for 1 d. Hgb = 8.6 g/dL	EGD: Soft, broad-based, 5 cm × 3 cm mass on greater curvature of stomach. Two ulcers on mass. Positive cushion sign. Abdominal CT: 4.5 cm × 3.0 cm gastric mass with attenuation of fat projecting into lumen. Doppler-assisted EUS: submucosal mass of mixed echogenicity	Greater curvature of stomach Surgical resection, 4.8 cm × 3.2 cm, mature adipocytes with ulceration and necrosis of overlying mucosa	Uneventful recovery. Unremarkable EGD at 6 mo of follow-up

Kumar *et al*[8], 2015	72 y. o. previously healthy M presented with presyncope associated with diaphoresis and pallor. Rectal exam revealed melena. Hgb = 9.9 g/dL	Abdominal CT: 4.3-cm-wide polypoid mass in antrum consistent with gastric lipoma. EGD: large, submucosal mass in gastric antrum with central ulcer with overlying clot. Ulcer injected with dilute epinephrine	Laparotomy: Gastrostomy with wide excision of antral lesion along anterior wall. Pathology: lipoma	Good postoperative recovery and discharged 3 d after surgery
López Cano *et al*[9], 1991	76 y. o. M with recent NSAID use, and hypertension presented with acute melena. Hgb = 6.8 g/dL	EGD: posterior wall of antrum 3.5-cm-wide lesion with overlying smooth mucosa. Central ulceration. Endoscopic biopsy: gastritis. Abdominal ultrasound with water-filled stomach: 4-cm-wide, echogenic submucosal mass	Partial gastrectomy Pathology: lipoma	No postoperative complications
Myint *et al*[10], 1996	54 y. o. F presented with hematemesis and melena for 1 wk. BP = 70/50 mmHg. Benign abdominal exam. Hgb = 4.0 g/dL.	EGD: 4 cm × 3 cm ulcerated submucosal mass in antrum Endoscopic biopsies: nondiagnostic. Abdominal CT: gastric mass with attenuation value of lipoma	Laparotomy: 6 cm × 6 cm mass in posterior wall of gastric antrum with central ulceration. Pathology: lipoma	Patient alive with no evident disease 6 mo after surgery
Ortiz de Solórzapo Aurusa *et al*[11], 1997	60 y. o. F. PMH: vitiligo, acute pancreatitis, duodenal ulcer presented with melena, postprandial pain, nausea, vomiting and early satiety. Pallor. Rectal exam: melena. Hgb = 12.8 g/dL	EGD: antral deformity. No active bleeding. Gastric volvulus? Abdominal USD: 5.8 cm × 3.4 cm pedunculated antral mass intussuscepting into duodenum. Abdominal CT: 4 cm × 3 cm × 3-cm-wide, well-defined, submucosal mass	Surgery; Underwent partial gastrectomy for antral mass intussuscepting into duodenum. Pathology: lipoma	Did well for 6 mo of follow-up
Paksoy *et al*[12], 2003	71 y. o. M with acute hematemesis and melena. BP = 110/70 mmHg, Pulse = 100/min Hematocrit = 27%	EGD: 4 cm-wide mass with superficial ulcer on posterior gastric wall. Endoscopic biopsies: "benign" lesion Abdominal CT: 4 cm lesion of lipid density in inferioposterior wall of stomach	Inferioposterior wall of stomach Surgery: laparoscopic transgastric resection of 4 cm intramural lipoma Pathology: intramural lipoma	Discharged 6 d postoperatively without complications
Pérez Cabañas *et al*[13], 1990	73 y. o. M presented with melena and hematemesis for 2 d. Recent NSAID use. PMH: hypertension. Physical exam: pallor, rectal exam-melena. Hgb = 8.6 g/dL. Transfused 5 units of packed erythrocytes	EGD: gastric mass on posterior wall and greater curve with superficial overlying ulcer, small hiatal hernia. Abdominal ultrasound: normal stomach. UGI series: large filling defect, from submucosal lesion	Surgery: Wedge resection for 5 cm × 4 cm submucosal mass Pathology: ulcerated lipoma	Did well after surgery
Priyadarshi *et al*[14], 2015	46 y. o. M with melena for 1 yr. Palpable, soft epigastric lump. Mild epigastric tenderness Hgb = 5 mg/dL; coagulation parameters and chemistry WNL	EGD: large mass arising from posterior wall antrum with superficial ulceration. Unable to traverse pylorus due to obstruction. Abdominal CT: huge mass with lobulated surface projecting into gastric lumen with density consistent with fat. Tumor extended into pylorus and caused gastric outlet obstruction	Posterior wall of gastric antrum Laparotomy: Billroth I partial gastrectomy; 14 cm × 11 cm × 5 cm sessile broad based submucosal lipoma; path = mature adipocytes	No reported complications
Rao *et al*[15], 2013	60 y. o. M presented with melena, fatigue and pallor. Hgb = 7.2 g/dL	EGD: large, smooth, submucosal bulge along lesser curvature of stomach. Contrast enhanced abdominal CT: Well-defined, encapsulated, submucosal mass with attenuation of fat along lesser curvature of stomach	Laparotomy: large submucosal tumor excised *via* anterior gastrotomy Pathology: 15 cm × 12 cm submucosal tumor with a focal ulcer. Microscopy demonstrates submucosal lipoma	Presently asymptomatic
Regge *et al*[16], 1999	52 y. o. M presented with hematemesis and melena. Hgb = 5.5 g/dL	EGD: 3.5-cm-wide, round, pale-pink formation on anterior gastric antrum with oozing superficial ulcer. Hemostasis achieved with dilute epinephrine injection. Abdominal USD: 4-cm-wide hyperechoic antral lesion with distinct margins. Abdominal CT with IV contrast: 4-cm-wide, well-circumscribed, antral lesion with density of fat. Abdominal MRI: Confirmed fat-tissue signal in mass by hyperintensity on T1-weighted images and marked signal reduction on sequences performed with fat suppression	Laparotomy: Antrectomy and gastrojejunal anastomosis *via* a Roux-en-Y loop. Pathology: lipoma	N.A
Sadio *et al*[17], 2010	44 y. o. M with medical history of hypertension, obesity, and sleep-apnea, presented with fatigue and intermittent melena for 1 mo. Physical exam revealed pallor. Hgb = 7.8 g/dL	EGD: 4-cm-wide, yellowish, submucosal mass in gastric fundus with central overlying ulceration. EUS: hyperechoic submucosal mass. Abdominal CT: homogeneous, well-circumscribed mass in fundus with density of fat	Surgery: partial gastric resection Pathology: submucosal lipoma	Did well and discharged 10 d postoperatively

Singh *et al*[18], 1987	40 y. o. M with melena, pyrexia, chills, and weakness. BP = 100/70 mmHg, pulse = 106/min, temp = 39 ℃, abdomen-soft, nontender, no palpable mass. Hgb = 4.0 g/dL	EGD: huge polypoid tumor in gastric body along greater curve. Multiple small superficial ulcers in antrum EGD biopsies: Mildly inflamed, mature adipose tissue UGI series: large gastric tumor	Gastric body along greater curve Laparotomy: smooth mass in gastric body and antrum. Multiple small ulcerations. Underwent subtotal gastrectomy and gastrojejunostomy. Pathology: 18 cm × 10 cm × 10 cm encapsulated lipoma	Discharged 2 wk postoperatively. Asymptomatic for 1 yr.
Youssef *et al*[19], 1999	54 y. o. nonalcoholic F presented with melena and dizziness Physical exam: stable vital signs, abdominal tenderness without peritoneal signs. Hgb = 9.2 g/dL	EGD: submucosal protrusion with mucosal erosion along greater curvature in body and antrum Abdominal USD: homogeneous, hyperechoic mass in submucosa of posterior gastric wall. Abdominal CT: homogeneous, 5.1 cm × 3.7 cm lesion with density of fat in posterior gastric wall	Laparotomy: with full-thickness resection of lesion Pathology: 5.2 cm × 3.8 cm × 3.2 cm submucosal lipoma	Uneventful recovery
Abdominal pain				
Alberti *et al*[20], 1999	11 y. o. F with periumbilical and RLQ abdominal pain for 3 yr. Outpatient UGI series revealed multiple filling defects in gastric antrum and body. Normal physical examination. Abdomen was soft with no palpable mass. No fecal occult blood Normal routine blood studies. Normal iron studies	EGD: multiple, large, soft, masses protruding into gastric body and antrum with normal overlying mucosa. Gastric biopsies: normal mucosa. Abdominal USD: multiple, homogeneous, well-encapsulated, submucosal masses with attenuation characteristic of fat. Abdominal MRI: solid, hyperintense formations with signal characteristic of fat in gastric body and antrum. Percutaneous transgastric ultrasound guided biopsy: features of lipoma with mild inflammatory infiltrate	Gastric body and antrum. No treatment because became asymptomatic	"Pain progressively relieved" Follow-up MRI of abdomen: no change
Hamdane *et al*[21], 2012	51 y. o. M with epigastric pain N.A	EGD: soft, large, ulcerated, submucosal mass in antrum Endoscopic biopsies: nonspecific inflammation of gastric mucosa. Abdominal CT: Round, well-circumscribed, low-attenuation, 9-cm-wide, gastric mass	Surgery: total gastrectomy. Pathology: 9 cm × 7.5 cm × 5 cm., mature adipocyte proliferation with variation of cell size in a fibro-myxoid background. Immunohistochemistry: positive to anti-HGMA2, but not S-100, or CD34, No MDM2 or CDK4 amplification, consistent with lipoma	Uneventful recovery. No symptoms at 1 yr follow-up
Neto *et al*[22], 2012	63 y. o. M history of dyslipidemia, and hypertension with upper abdominal pain. Physical exam reveals a palpable, moveable upper abdominal mass Normal routine laboratory tests	Abdominal USD: large echoic mass compatible with an expansive lesion in gastric antrum. EGD: large bulging mass in posterior gastric wall with three ulcerated areas Endoscopic biopsies: necrotic mucosa Abdominal CT: well-defined, homogeneous, oval mass located within the posterior gastric wall that compressed descending duodenum and had the density of fat	Posterior gastric wall. Laparotomy with a subtotal gastrectomy and D1 lymphadenectomy with Roux-en-Y reconstruction: 12 cm × 8 cm × 6 cm, lipoma with mature, well differentiated adipocytes surrounded by a fibrous capsule with 3 ulcerative lesions of 0.5 cm, 1 cm, and 1.4 cm	Uneventful recovery with discharge 7 d postoperatively
Ramaraj *et al*[23], 2012	52 y. o. M with dyspepsia, anorexia, and early satiety for 6 mo. Gastric ulcer 5 yr earlier. Iron deficiency anemia: Hg = 11.5 g/dL, ferritin = 5 ng/mL	Colonoscopy: within normal limits. EGD: Extrinsic indentation in distal stomach with smooth overlying mucosa. Endoscopic biopsy: normal mucosa CT abdomen: 15 cm × 14 cm fatty tumor in distal stomach	Antrum Subtotal gastrectomy: Submucosal antral lipoma with central ulceration	No postoperative complications. Asymptomatic at 4 wk of follow-up
Zak *et al*[24], 2006	58 y. o. M with intermittent upper abdominal discomfort, early satiety, smoking, hyperlipidemia, obesity, PTSD, and depression. Has iron deficiency anemia	EGD: 10 cm × 6 cm smoothly lobulated, submucosal mass in gastric antrum along greater curvature. Chronic inflammation and intestinal metaplasia of gastric mucosa. EUS: hypoechoic submucosal mass surrounded by a hyperechoic layer in posterior wall of stomach, consistent with encapsulated lipoma. Abdominal CT: homogeneous, round, sharply-defined, encapsulated, submucosal lesion with characteristic density of fat	Gastric antrum along the greater curvature Laparotomy: resection only of the encapsulated mass Pathology: 10 cm × 6 cm lipoma	Uneventful recovery with discharge on day 7. Follow-up abdominal CT 2 mo later revealed no abnormalities
Predominantly nausea and vomiting or obstructive symptoms				

Aslan *et al*[25], 2015	77 y. o. M with nausea and vomiting, and dyspepsia. Complete blood count and comprehensive metabolic panel: WNL	EGD: submucosal mass with normal overlying mucosa extending into antrum along lesser curve	Endoscopic submucosal resection of 9-cm-long lipoma with an intact capsule	Discharged after 3 d. Resolution of symptoms at 6 mo of follow-up. Repeat endoscopy did not reveal a mass
Lin *et al*[26], 1992	77 y. o. F with nausea, vomiting, abdominal pain for 3 wk and 7-kg-weight-loss. Dehydrated and generalized mild abdominal tenderness. Rectal exam: fecal occult blood	UGI series: large polypoid gastric mass intussuscepting into duodenum. Abdominal USD: suspected intussusception. EGD: inadequate examination. Differential of gastric torsion *vs* intussusception	Laparotomy: large necrotic polypoid intussuscepting mass arising in stomach. Polyp resected at its base. Pathology: large polypoid lipoma	Ultimately recovered and was discharged
Mouës *et al*[27], 2002	72 y. o. M with anorexia, early satiety, nausea, and involuntary weight loss. No overt GI bleeding. Left lung lobectomy for bronchial lung cancer 10 yr earlier. Hemoglobin = 4.7 g/dL	EGD: gastric mucosal hypertrophy extending into duodenum. Abdominal USD: hyperechoic mass in small intestine, consistent with lipoma, with likely intussusception. CT abdomen: low attenuation intraluminal tumor compatible with small intestinal lipoma	Laparotomy: large pedunculated tumor intussuscepting into jejunum. Mass reduced back into stomach. Gastrostomy revealed 10 cm × 5 cm superficially ulcerated gastric lipoma. Mass excised. Pathology: mature adipose tissue	Uneventful recovery
Nasa *et al*[28], 2016	56 y. o. F with dyspepsia and occasional vomiting for 1 yr. Mild epigastric tenderness	EGD: smooth 5-cm-wide antral bulge with overlying normal mucosa. Positive cushion sign. Endoscopic biopsy: chronic active gastritis from Helicobacter pylori. EUS: homogeneous, hyperechoic, mass arising from layer 3 of gastric wall, compatible with lipoma. Abdominal CT: homogeneous, 6-cm-wide, oval mass in antropyloric region, with density of fat	Antrum and pylorus along lesser curve Laparotomy: Excision of 6 cm wide, encapsulated tumor along lesser curve of stomach	Did well and discharged. Asymptomatic at 6 mo
Treska *et al*[29], 1998	61 y. o. M with intermittent vomiting for several days. History of gastric ulcer N.A	UGI series: spherical, smooth, 4.0 cm × 4.5 cm defect in gastric antrum. EGD: protruding, yellowish tumor in prepylorus. Two ulcers above tumor. Abdominal ultrasound: 7 cm × 6 cm × 5 cm echogenic defect in wall of gastric antrum. Abdominal CT: prepyloric intramural lipoma	Gastric antrum. Laparotomy: 7.0 cm × 6.0 cm tumor in prepylorus. Tumor resection of lipoma with performance of Billroth Ⅱ	Discharge 12 d postoperatively. No GI symptoms 8 mo after surgery
Lipoma discovered incidentally in work-up for other condition				
Al Shammari *et al*[30], 2016	41 y. o. M presented for morbid obesity with a BMI of 43.9 kg/m^2 and history of obstructive sleep apnea. Normal routine blood tests	Abdominal ultrasound: liver span of 18.8 cm. EGD: rounded 3 cm × 3 cm mass in antrum with normal overlying mucosa. Positive cushion sign. Abdominal CT: 3.5 cm × 3.0 cm lesion in stomach suspicious for lipoma	Antrum. Laparoscopy: Intragastric submucosal mass excised from inside stomach after gastrostomy. Sleeve gastrectomy then performed for morbid obesity. Pathology: 4 cm × 3 cm × 2 cm lipoma	Discharged 4 d postoperatively. Asymptomatic at 2 wk of follow-up
Hyun *et al*[31], 2002	22 y. o. M who underwent abdominal CT as preoperative evaluation of retroperitoneum before orchiectomy for testicular cancer. N.A	Abdominal CT: large gastric mass with attenuation of fat projecting into gastric lumen. EGD: large, soft, sessile mass on greater curve of stomach with overlying pink mucosa. Positive cushion sign. Endoscopic biopsies: normal mucosa. EUS: Submucosal mass with less echogenicity than expected for lipoma	Surgical resection: 12 cm × 9 cm × 2.5 cm mobile mass resected. Pathology: Submucosal gastric lipoma	Doing well at 2 mo follow-up
López-Zamudio *et al*[32], 2015	59 y. o. M who underwent abdominal CT performed during episode of acute alcoholic pancreatitis revealed probable pyloroduodenal intussusception of a tumor with attenuation suggestive of fat. Hgb = 9.3 g/dL	EGD: 8 cm long polypoid mass impeding flow near pylorus. EGD biopsy: gastritis and incomplete intestinal metaplasia. Repeat EGD: greater curve posterior wall large pedunculated polyp with central ulceration Repeat EGD biopsies: chronic gastritis, focal ulceration intestinal metaplasia and *Helicobacter pylori* infection. EUS: 5.6 cm × 4.9 cm mass in gastric antrum in muscular layer	Surgery: 5 cm × 5 cm tumor in anterior wall of gastric antrum. Underwent antroduodenectomy with gastroduodenal anastomosis and Roux-en-Y	No postoperative surgical complications. Asymptomatic at 18 mo of follow-up

PMH: Previous medical history; GI: Gastrointestinal; y.o.: Years old; M: Male; F: Female; Hgb: Hemoglobin; BP: Blood pressure; EGD: Esophagogastroduodenoscopy; CT: Computerized tomograph; EUS: Endoscopic ultrasound; UGI: Upper gastrointestinal; USD: Ultrasound; N.A: Not applicable; NSAID: Nonsteroidal anti-inflammatory drug; WNL: Within normal limits; IV: Intravenous; MRI: Magnetic resonance imaging; RLQ: Right lower quadrant; PTST: Post traumatic stress disorder; BMI: Body mass index.

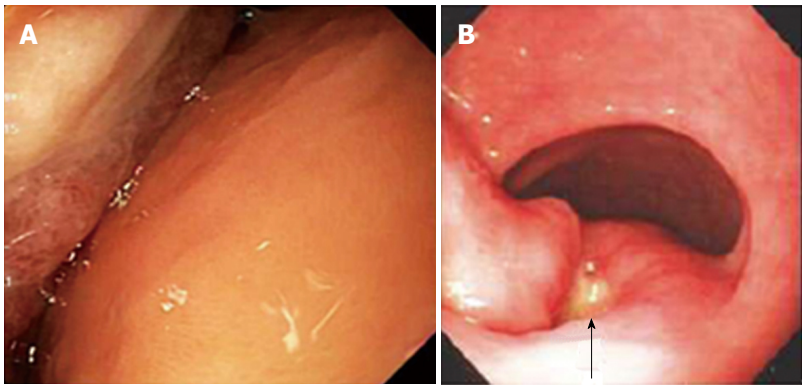

Figure 1 Findings at esophagogastroduodenoscopy in two patients with giant gastric lipomas. A: Patient 1. Esophagogastroduodenoscopy (EGD) in a 63-year-old male who presented with melena and a hemoglobin decline to 6.2 g/dL that required transfusion of 2 units of packed erythrocytes, showing the distal body and antrum with a huge mass folded upon itself occupying most of the lumen and an 8 mm wide, nonbleeding, acute mucosal ulcer without stigmata of recent hemorrhage embedded deep in the valley (fold) between the right and left parts of the mass. The ulcer was attributed to friction from the opposing surface. The mass was 13-cm-wide, submucosal, yellowish, and covered by smooth mucosa except for focal ulceration, findings consistent with a gastric lipoma; B: Patient 2. EGD in a 78-year-old-woman, who presented with melena for 3 d, orthostatic dizziness, and a hemoglobin decline to 7.1 g/dL requiring transfusion of 2 units of packed erythrocytes, revealed an acute 1-cm-wide prepyloric ulcer (arrow) with a white exudate but without stigmata of recent hemorrhage between the right and left lobes of a large, well-demarcated, submucosal, mass covered by otherwise normal, superficial mucosa. This endoscopic photograph shows only a part of the mass.

analyzed esophagogastroduodenoscopies (EGDs) at two large hospitals.

MATERIALS AND METHODS

The literature was systematically reviewed using PubMed for articles published since 1980 with the following medical subject heading (MeSH) or keywords: ("giant lipoma") AND (gastric) OR [("lipoma") and ("gastric") and ("bleeding")]; and by reviewing the section on gastrointestinal lipomas in standard pathology textbooks or monographs. Two authors independently reviewed the literature, and decided by consensus which articles to incorporate in this review. After reviewing one case from 1974[5], cases reported before 1980 were selectively excluded because the preoperative evaluation at the time frequently used relatively obsolete tests such as UGI series and often lacked currently mandatory tests such as EGD. Four case reports, written in Spanish[9,11,13,32], were professionally translated into English. Case reports of large gastric adenomas which did not satisfy the minimal size criteria of giant gastric lipomas (≥ 4 cm) were systematically excluded[3,34]. A video publication was excluded because clinical details were not reported[35]. A clinical series of 16 gastric lipomas were excluded because this series lumped together medium-sized and giant lipomas[36].

Computerized review of the pathology records at William Beaumont Hospitals at Royal Oak and at Troy, Michigan from January 2005-December 2015 using the computerized system of PowerPath (Tamtron) and SOFTPath with the software terms ("lipoma" or "lipomas") AND ("gastric" or "stomach") revealed 2 cases of giant gastric lipomas. Computerized review of the EGD reports using Provations did not reveal any further cases. These 2 cases were thoroughly reviewed based on medical records, including re-review of the original endoscopic photographs by an expert endoscopist, radiologic images by an expert radiologist, and pathologic slides by an expert pathologist. This dual case report received exemption/approval by the IRB at William Beaumont Hospital, Royal Oak, on October 17, 2016.

Illustrative case reports

Case 1: A 63-year-old, nonalcoholic, man with a medical history of hypertension treated with lisinopril, amlodipine, and nifedipine, and hyperlipidemia treated with lovastatin, presented with epigastric pain, early satiety, and involuntary 10-kg-weight-loss during the last 6 mo, and melena and dyspnea on exertion for 3 d. The vital signs were stable, with a blood pressure of 144/74 mmHg, and pulse of 87/min. The abdomen was soft, nontender, and without hepatosplenomegaly or palpable masses. Rectal examination revealed melena. The hemoglobin was 6.2 g/dL, blood urea nitrogen was 27 mg/dL, and creatinine was 1.40 mg/dL. He had 496000 platelets/mL, a normal international normalized ratio (INR), and normal partial thromboplastin time (PTT). He was transfused two units of packed erythrocytes.

EGD revealed a 13-cm-wide submucosal, yellowish, gastric mass primarily in the antrum, covered by smooth mucosa except for focal ulceration (Figure 1A), and exhibiting the pillow sign, of indentation of the mass with moderate pressure applied *via* a closed forceps[37,38]. Microscopic examination of multiple mucosal biopsies of the ulcer margin revealed superficial ulceration, granulation tissue, and no malignancy. Abdominal computerized tomography (CT) revealed a well-circumscribed, homogeneous, 13.4 cm × 8.4

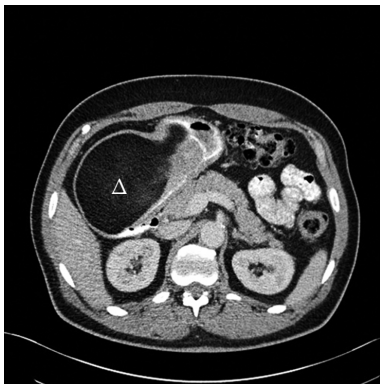

Figure 2 Abdominal computerized tomography findings in patient 1. A 63-year-old male (patient 1) presented with acute melena and hemoglobin decline to 6.2 g/dL, and esophagogastroduodenoscopy revealed a huge, submucosal mass with a smooth overlying surface and exhibiting the pillow sign characteristic of a submucosal lipoma. Illustrated abdominal computerized tomography shows an approximately 13.4 cm × 8.2 cm × 8.4 cm mostly homogeneous, hypodense mass with a characteristic attenuation of fat (-90.2 Hounsfield units) extending from proximal gastric body through entire antrum. The normal-appearing very proximal stomach is filled with oral contrast without a mass, and leads to a very narrow, compressed, distal and dorsal, gastric channel containing oral contrast that passes into the duodenum. Triangle: antral giant gastric lipoma which has the characteristic hypodense attenuation of fat.

cm × 8.2 cm mass, with attenuation characteristic of fat, arising from the gastric antrum and producing a mass effect on the proximal duodenum (Figure 2). A 7-mm-wide lesion in the body of the pancreas was also suspected to be a lipoma based on its characteristic attenuation. The patient underwent laparotomy due to the recent bleeding of the giant lipoma. It was resected *via* subtotal gastrectomy extended by partial bulbar duodenectomy due to lipoma extension into duodenal bulb, with Billroth Ⅱ reconstruction. Gross pathology revealed a homogeneous, soft, submucosal mass with a cut surface exposing yellowish, greasy tissue, measuring 14.5 cm × 8.7 cm × 7.5 cm (Figure 3A), which microscopically revealed lipoma (Figure 4A). Immunohistochemical staining revealed positivity for CD34 (Figure 4B), a finding highly consistent with a spindle cell variant lipoma[39]. The patient was discharged 8 d postoperatively, and had no complications during 8 wk of follow-up.

Case 2: A 78-year-old, nonalcoholic, woman with a medical history of atrial fibrillation, peripheral neuropathy, hypertension, dyslipidemia, diabetes, chronic renal insufficiency, and hysterectomy for uterine fibroids, presented with melena for three days, associated with fatigue and orthostatic dizziness. Medications included warfarin, furosemide, metoprolol, diltiazem, atorvastatin, pioglitazone, amitriptyline, and glimepiride. Physical examination revealed stable vital signs, with a blood pressure of 124/67 mmHg, and pulse of 68/min. There was a heart murmur, and bilateral 3+ lower extremity edema. The abdomen was soft, and non-tender, with normoactive bowel sounds, and no organomegaly. Rectal examination revealed melena. The hemoglobin was 7.1 g/dL, INR was 4.9, platelet count was 306000/mL, and PTT was 42.5 s. The blood urea nitrogen was 44 mg/dL and creatinine was 1.8 mg/dL. An electrocardiogram revealed atrial fibrillation without acute ischemic changes.

She was transfused 2 units of packed erythrocytes and 2 units of fresh frozen plasma. Two-dimensional echocardiography revealed mild mitral valve regurgitation, moderate-to-severe right atrial dilatation, and severe tricuspid valve regurgitation. Abdominal CT revealed a submucosal, antral, gastric mass measuring 9.5 cm × 6.0 cm × 4.5 cm. EGD revealed a large, focally ulcerated, smooth, antral gastric mass, exhibiting the cushion sign (Figure 1B). Microscopic examination of multiple mucosal biopsies of the ulcer margin revealed superficial ulceration, granulation tissue, and no malignancy. The patient underwent surgery due to the recent bleeding of the giant lipoma. At laparotomy, the submucosal mass was excised by distal gastrectomy. Gross pathology of the resected mass revealed a relatively homogeneous, 9.0 cm × 6.0 cm × 4.5 cm, focally ulcerated, mass with a greasy, tan-yellow cut surface (Figure 3B), which microscopically was a lipoma (Figure 4C and D). The patient was discharged 5 d postoperatively with no further GI bleeding[37-39].

RESULTS

Systematic literature review revealed that giant gastric lipomas are rare, with only 32 cases reported since 1980 (Table 1), and only 2 cases currently identified among 117110 EGDs performed during 11 years at William Beaumont Hospital, Royal Oak, one of the five largest hospitals in the United States, and at William Beaumont Hospital, Troy. The 32 reported patients were on average 54.5 ± 17.0 years old. Thirty were adult patients, and two were pediatric patients. Twenty-two were male, and 10 were female. Twenty-one patients had an ulcerated mass. The lipomas averaged 7.9 cm ± 4.1 cm in maximal dimension. Lipoma locations included antrum-17, body and antrum-4, antrum intussuscepting into small intestine-3, gastric body-2, fundus-1, and unspecified-5. This data confirms previous reports that giant gastric lipomas most commonly occur in the antrum[40]. Intramural locations included submucosal-22, subserosal-2, and unspecified-8. This data confirms previous reports that giant gastric lipomas are generally submucosal, but occasionally subserosal[22,36].

Nineteen patients presented with acute UGI bleeding, including melena-11, hematemesis and melena-7, and hematemesis-1. Giant lipomas can ulcerate and bleed secondary to venous stasis[14], friction and trauma

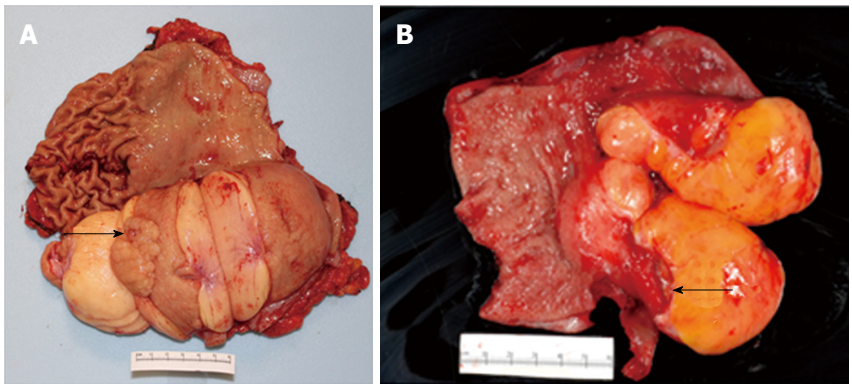

Figure 3 Gross pathologic findings in gastrectomy specimens in two patients with giant gastric lipomas. A: Patient 1. Patient 1 presented with acute melena and hemoglobin decline and had an ulcer detected at esophagogastroduodenoscopy (EGD) within a huge, lipomatous gastric mass. Gross pathologic view of the gastrectomy specimen after it is opened to expose the luminal surface shows a well-circumscribed, lobulated, 14.5 cm × 9.0 cm × 7.5 cm lipomatous mass extending from the gastric body (left) to antrum (right). A small ulcer (round depression, arrow) is present on the mucosa overlying the lipomatous mass. Normal gastric rugae are present above the mass on the upper left, but have been effaced on the upper right, likely because of chronic compression/pressure from the giant lipomatous mass located below (on the contralateral gastric wall before opening the stomach). Vertical incisions show a homogeneous yellow-tan cut surface, indicative of a lipomatous tumor; B: Patient 2. Patient 2 presented with melena for 3 d, orthostatic dizziness, and a hemoglobin decline to 7.1 g/dL requiring transfusion of 2 units of packed erythrocytes and had at EGD a large, yellowish, smooth, well-circumscribed antral mass. Gross pathologic view of distal gastrectomy specimen after it is opened to expose the luminal surface shows normal gastric antral tissue at left and a lobulated, well-circumscribed, yellow-tan, 9.0 cm × 6.0 cm × 3.5 cm lipomatous tumor at right, with a deep, clean-based, ulcer (arrow) on the mucosa overlying the mass.

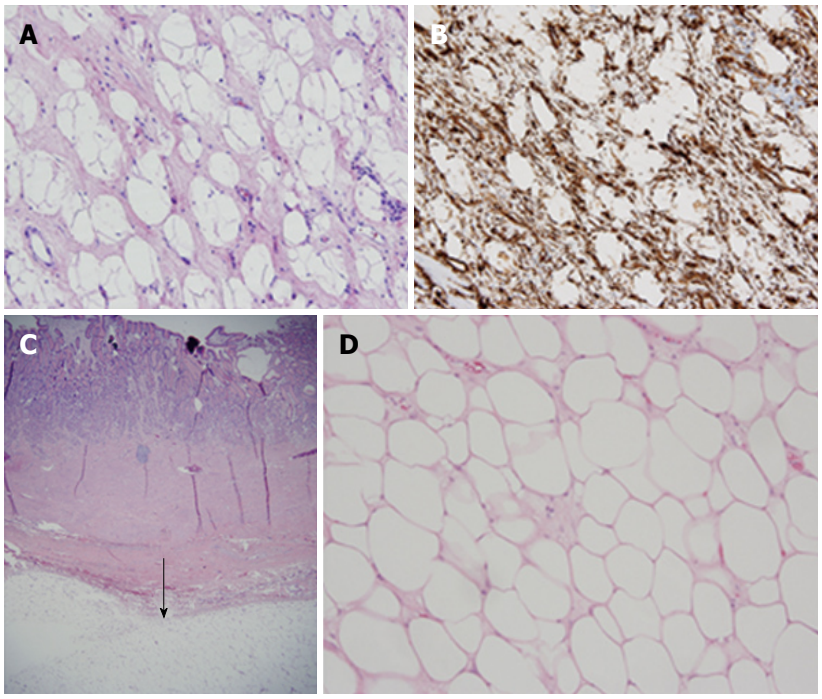

Figure 4 Histopathologic findings in gastrectomy specimens in two patients with giant gastric lipomas. A: Patient 1-standard histochemistry. Medium power photomicrograph of a hematoxylin and eosin stain of a tissue section from the resected gastric mass in patient 1 reveals large adipocytes filled with clear, homogeneous, cytoplasm and tiny, compressed, peripheral nuclei. No lipoblasts are detected. Note the spindle-shaped stroma surrounding the adipocytes, findings consistent with spindle cell lipoma, as proven by immunohistochemistry (B); B: Patient 1-immunohistochemistry. Medium power photomicrograph of immunohistochemistry, using an antibody to CD34, reveals within tumor in patient 1 extensive staining in a spindly pattern of the stroma surrounding characteristically clear adipocytes, a characteristic staining pattern for spindle-shaped lipoma; C: Patient 2-standard histochemistry-low power. Low power photomicrograph of a hematoxylin and eosin stain of a tissue section from resected gastric mass in patient 2 reveals a well-circumscribed, submucosal layer composed of adipocytes with clear cytoplasm (arrow) and scant loose, myxoid stroma; D: Patient 2-standard histochemistry-medium power. Medium power photomicrograph of a hematoxylin and eosin stain of a tissue section from resected gastric mass in patient 2 reveals sheets of large, adipocytes filled with clear, homogeneous, cytoplasm and tiny, compressed, peripheral nuclei, with scant loose, myxoid stroma. No lipoblasts are detected. These histologic findings are characteristic of lipomas.

Table 2 Risks factors for upper gastrointestinal bleeding among 32 patients with giant gastric lipomas

Parameter	mean ± SD of parameter in patients with bleeding	mean ± SD of parameter in patients without bleeding	Patients with bleeding: *n* with ulcer/ total *n* (% with parameter)	Patients without bleeding: *n* with parameter/total *n* (% with parameter)	*P* value	OR	95%CI	Statistical test
Continuous variables								
Patient age	54.9 ± 15.5 yr	53.8 ± 19.6 yr	-	-	0.87	NA	NA	Student's *t* test
Lipoma size	7.1 cm ± 4.4 cm	9.3 cm ± 3.1 cm	-	-	0.16	NA	NA	Student's *t* test
Dichotomous variables								
Male sex	-	-	12/19 (63.2)	10/13 (76.9)	0.47	0.51	0.08-3.17	χ^2 test
Ulcer overlying lipoma	-	-	16/19 (84.2)	4/13 (30.8)	0.004	12.0	1.72-101.9	Fisher's exact test

UGI: Upper gastrointestinal; *P*: Probability; NA: Not available.

of the lipoma tip against the wall contralateral to the lipoma attachment site, or, least likely, from outgrowing their blood supply. Among 19 patients presenting with acute GI bleeding, the hemoglobin on admission averaged 7.5 g/dL ± 2.8 g/dL (unavailable in 2 patients). Symptoms in the 19 patients included: weakness/fatigue-6, abdominal/epigastric pain-4, nausea and vomiting-4, epigastric fullness/early satiety-3, dizziness/presyncope-2, and belching-1. Signs included: pallor-7, epigastric tenderness-3, epigastric mass-3, tachycardia-2, and one each with diaphoresis or hypotension. Among four analyzed variables including age, sex, lipoma size, and lipoma ulceration, only lipoma ulceration was statistically significantly different (more common) in patients presenting with UGI bleeding, than in patients with other presentations ($P = 0.004$; Table 2). This difference emphasizes the importance of lipoma ulceration in the pathogenesis of bleeding.

Five patients presented predominantly with abdominal pain, without acute UGI bleeding, including 2 presenting with iron deficiency anemia. Additional symptoms in these 5 patients included early satiety-2, and anorexia-1. Five patients presented with nausea and vomiting. These patients had additional symptoms including dyspepsia/abdominal pain-3, weight loss-2, anorexia-1, and early satiety-1. Three patients had asymptomatic giant gastric lipomas incidentally detected: by EGD before bariatric surgery for morbid obesity; by abdominal CT in the evaluation of testicular cancer; and by abdominal CT for severe acute pancreatitis.

Thirty-one of the 32 patients underwent EGD (one other patient, see Methods section). EGD is standardly performed preoperatively to characterize the anatomy and show characteristic endoscopic features of lipomas. However, EGD frequently fails to obtain diagnostic tissue due to failure of superficial mucosal biopsies to reach submucosa. Among 12 patients in whom endoscopic biopsy results were reported in the present review, only 1 (8.3%) had lipoma diagnosed pathologically by endoscopic biopsies[18]. Pathologic findings in the other 11 reported biopsy specimens included chronic or nonspecific inflammation, gastritis, and normal or necrotic tissue. Repeated biopsies at the same site (well technique) may increase somewhat the diagnostic yield of endoscopic biopsies. Repeated or deep biopsies at EGD may expose yellow fat from the lipoma, a finding called the "naked fat" sign[41,42]. In the tenting sign observed at EGD the superficial mucosa retracts from the submucosal mass when it is grasped with a forceps because the submucosal lipoma has a fibrous capsule and does not infiltrate into the mucosa. Submucosal lipomas tend to be smooth except for focally ulcerated areas.

CT is currently the standard imaging modality. Lipomas are identified by having an attenuation ranging from -70 to -120 Hounsfield units, characteristic of fat density[20]. Twenty (95.2%) of 21 patients undergoing abdominal CT had CT findings highly suspicious for lipoma, and the other one had diagnostically helpful findings. CT findings consistent with a lipoma include: a well-circumscribed, submucosal, and homogeneous, mass with an attenuation characteristic of fat. Seven patients underwent upper gastrointestinal series which revealed mass size, mass location, and a smooth superficial layer, but did not show characteristic features of lipomas[4-6,13,18,26,29]. All 7 patients undergoing UGI series were reported in publications from 1998 or before, whereas only 4 of 21 patients undergoing CT were reported in publications from 1998 or before (17 CTs reported in publications from 1999-2016) ($P < 0.00001$, OR > 3.53, Fisher's exact test). This difference is consistent with replacement of UGI series by EGD and abdominal CT which provide superior characterization. In 9 (82%) of 11 cases abdominal ultrasound showed features suspicious for lipomas of a well-demarcated, submucosal, hyperechoic lesion, but the lesion was missed in 2 cases[4,9,11,16,19,20,22,26,27,29,30]. These results are consistent with abdominal ultrasound being a cheaper, but less definitive test than abdominal CT for giant gastric lipomas.

In 4 (80%) of 5 cases, endoscopic ultrasound (EUS) showed characteristic findings of a lipoma of a hyperechoic, well-localized, submucosal mass, but in one case the findings were atypical[17,24,28,31,32]. EUS is useful to identify the primary wall layer of lipomas[22,43]. The currently reported findings are consistent with EUS being an important adjunct test when abdominal CT is non-diagnostic, when a tissue diagnosis is needed preoperatively because of non-diagnostic EGD biopsies, or prior to endoscopic mucosal resection. Both patients undergoing abdominal magnetic resonance imaging (MRI) had important findings showing tumor anatomy and exhibiting signals characteristic of fat[16,20].

Therapy was successful in all 32 patients including laparotomy with full-thickness resection of gastric wall containing tumor either *via* wedge resection, partial gastrectomy, Billroth I resection or other surgery-26; laparotomy with enucleation-2; laparoscopic transgastric resection-2; endoscopic mucosal resection-1, and successful laparotomy with polypectomy after unsuccessful, attempted endoscopic polypectomy-1. No patient suffered major postoperative or postprocedural complications. No patient died from GI bleeding from the lipoma, from the surgery, or from endoscopic therapy.

When GI bleeding or gastric obstruction are associated with a large, ulcerated gastric mass, gastric malignancy may be suspected. It is critical to preoperatively exclude liposarcomas from giant lipomas because liposarcomas require further genetic analysis of pathologic specimens, and chemotherapy after surgical resection[44]. Lipomas have well-demarcated margins on radiologic imaging due to the presence of a fibrous capsule. They are well-differentiated, devoid of lipoblasts, and grow slowly. Liposarcomas have a densitometry close to normal submucosal tissue at abdominal CT[20,45], and are definitively diagnosed by MDM2 and CDK4 gene amplification[21,26]. Other tumors in the differential diagnosis of giant lipomas include GI stromal tumors, such as leiomyoma and fibroma; and rarely intramural tumors, such as neurilemoma, adenomyoma, Brunner's gland adenoma, and heterotopic pancreas[22].

Treatment for lipomas is not standardized. They are often resected endoscopically when < 4-6 cm, and surgically when > 6 cm[14,22], but endoscopy has been used to resect up to 9-cm-wide gastric lipomas[25]. Lipomas may sometimes be resected by enucleation because they are encapsulated. Resection *via* subtotal gastrectomy entails much greater morbidity from potential complications of anastomotic leakage, duodenal stump rupture, obstruction, hemorrhage, decreased acid production, delayed gastric emptying, gastroesophageal reflux, and vitamin B12, folate, iron, or calcium deficiencies[24].

DISCUSSION

The current literature review demonstrates characteristic findings of giant gastric lipomas at EGD, abdominal CT, EUS, and immunohistochemistry, as summarized in Table 3[3,8,14,18,20,22,36-38,40-42,47-52]. The two currently reported cases illustrate characteristic features of giant gastric lipomas: frequently presenting with acute UGI bleeding which is often severe and life-threatening, characteristic endoscopic features, characteristic CT findings, pathologic findings indicating benignity, and excellent post-operative prognosis with rare major morbidity or mortality.

The current case reports are limited by retrospective analysis, and by only reporting 2 cases due to disease rarity. The current literature review is likewise limited first, by consisting of single case reports due to syndrome rarity; and second, by retrospectively reporting of case reports. Individually reported cases may be subject to selection bias with preferential reporting of more clinically dramatic or more successful therapeutic interventions. Third, cases from different centers reported somewhat variable clinical data, such as variable follow-up and variable imaging tests (*e.g.,* abdominal CT *vs* MRI). Fourth, the evaluation of lipomas has evolved over time due to development of better diagnostic tests. This effect was minimized by excluding cases reported before 1980. Fifth, imaging tests were interpreted by various radiologists and pathology specimens were interpreted by various pathologists at various hospitals in the prior case reports.

In conclusion, this systematic literature review provides a comprehensive analysis to help optimize the evaluation and management of suspected giant gastric lipomas. CT and EGD are the standard tests to evaluate suspected giant gastric lipomas. When giant gastric lipomas are identified at abdominal CT by characteristic findings of a homogeneous, well-circumscribed, submucosal mass with characteristic attenuation of fat, EGD with biopsies should be performed, but the endoscopic biopsies may be non-diagnostic. EUS with deep biopsies may be performed to obtain a definitive diagnosis if biopsies from EGD are non-diagnostic. Liposarcoma should be excluded by cytogenetic analysis when necessary. If lipoma is confirmed, endoscopic resection of only the lipoma and its fibrous capsule may be feasible for small-to-moderate sized lesions, with subtotal gastrectomy reserved for especially large lipomas.

Table 3 Distinctive features of giant gastric lipomas

Test/technique/parameter	Distinctive characteristic	Pathophysiology	Ref.
Age	Average age = 54.5 ± 17.0 years old		Current Report
Gender ratio	Male-to-Female ratio approximately 2:1	Unknown (sexual hormones?)	Current Report
Lipoma size	Average maximal dimension = 7.9 cm × 4.1 cm		Current Report
Most common clinical presentation	19 of 32 presented with acute UGI bleeding	Postulated from ulcer at tip of lipoma caused by rubbing/trauma of tip against gastric wall contralateral to base of lipoma	Current Report
EGD	Smooth bulge covered by normal mucosa	Submucosal (or occasionally subserosal) location. No tumor invading mucosa due to benignity	Neto *et al*[22], 2012, Thompson *et al*[36], 2003
	Most commonly located in gastric antrum		Thompson *et al*[36], 2003, Menon *et al*[40], 2014
	Yellowish hue	Yellow color of adipose tissue in submucosa transmitted to mucosal surface	Menon *et al*[40], 2014, Chen *et al*[41], 2014
	Broad base	Rarely pedunculated	Singh *et al*[18], 1987
	Cushion or pillow sign: easily deforms like a cushion with mild pressure applied against it by an endoscopic probe (closed biopsy forceps).	Lipoma consists of soft, compressible tissue.	De Beer *et al*[37], 1975, Hwang *et al*[38], 2005
	Tenting sign: Mucosa easily retracts after it is grasped and gently pulled with a forceps	Mucosa separates from submucosa when gently pulled *via* forceps because lipoma has fibrous capsule and does not infiltrate into adjacent tissue	Priyadarshi *et al*[14], 2015
	Naked fat sign: repeated biopsies at same site reveals yellow fatty tissue	Multiple biopsies at same site (using well technique) exposes submucosal lipomatous tissue	Chen *et al*[41], 2014, Patrick *et al*[42], 2007
	Moderately frequent focal central ulceration of mucosa	Likely secondary to giant lipoma abutting and rubbing against contralateral gastric wall. Ischemia may also contribute to ulceration.	Kumar, *et al*[8], 2015, Thompson *et al*[36], 2003
Highly useful diagnostic test for lipomas	Typically strongly suggestive of diagnosis	Demonstrates anatomy of mass. Shows if ulcerated or intussuscepting mass. Characteristic findings: yellow hue, smooth overlying mucosa, relatively homogeneous, round margins. Exhibits pillow, tenting or naked fat signs.	Current Report
Endoscopic biopsies	Standard endoscopic biopsies usually reveal only normal mucosa and insensitive for pathologic diagnosis.	Standard endoscopic biopsies typically sample superficial mucosa and miss deeper submucosal lipoma.	Current Report, Neto *et al*[22], 2012
	Techniques to increase yield of endoscopic biopsies; use jumbo forceps for endoscopic biopsies; or well technique (repeated endoscopic biopsies at same mucosal site).	Repeated biopsies at same site permits sampling of deeper (submucosal) tissue	Wang *et al*[47], 2015
Abdominal CT	Submucosal mass	Typically submucosal, occasionally subserosal, and never mucosal.	Beck *et al*[3], 1997
	Well-circumscribed with well-defined edges	Characteristically has a firm fibrous capsule with no invasion through capsule due to benignity	Thompson *et al*[36], 2003
	Typically solitary	Multiple gastric lipomas are very rare	Park *et al*[48], 1999, Skinner, *et al*[49], 1983
	Homogeneous	Composed of homogeneous lipocytes	Park *et al*[48], 1999, Alkhatib *et al*[50], 2012
	Densitometry of -80 to -120 HU (Hounsfield units).	Characteristic of adipose tissue	Alberti *et al*[20], 1999
Highly useful as diagnostic test for gastric lipomas	Demonstrates characteristic findings in about 95% of cases.	Characteristic findings: well-circumscribed, submucosal, homogeneous mass with an attenuation characteristic of fat.	Current Report
EUS	In third layer of gastric wall	Typically submucosal (rarely subserosal)	Chen *et al*[43], 2011
	Hyperechoic (bright)		Alkhatib *et al*[50], 2012, Eckardt *et al*[51], 2012

EUS-guided needle biopsy or endoscopic mucosal resection	EUS guidance used to obtain diagnostic deep (submucosal) biopsies	Deep biopsies permit sampling of submucosal lipomas	Alkhatib *et al*[50], 2012, Karaca *et al*[52], 2010
Transcutaneous abdominal ultrasound	Not very useful for gastric lipomas.	Supplaned by abdominal CT or EUS for evaluating suspected gastric lipomas	Current Report
Upper gastrointestinal series	Mostly obsolete test	CT is a superior alternative	Current Report
Histopathology	Diagnostic features	Rounded, plump cells with abundant clear, homogeneous cytoplasm containing fat, eccentric nuclei, mature adipocytes with no lipoblasts, scant stroma, rare inflammatory cells.	Current Report
Imunohistochemistry	Reveals no MDM2 or CDK4 gene amplification.	Distinguishes lipoma from liposarcoma.	Shimada *et al*[45], 2006, Boltze *et al*[46], 2001
Immunohistochemistry	Lipoma stains positively for CD4	Indicates spindle-cell lipoma variant	Lau *et al*[39], 2015

UGI: Upper gastrointestinal; EGD: Esophagogastroduodenoscopy; CT: Computerized tomography; EUS: Endoscopic ultrasound.

COMMENTS

Background

Gastric lipomas are rare, constituting < 1% of all gastric tumors, and giant gastric lipomas (⩾ 4 cm) are extremely rare, with this systematic review identifying only 32 cases reported since 1980. Although small gastric lipomas are usually asymptomatic, giant gastric lipomas typically produce major, clinically important, symptoms from GI obstruction, tumor ulcers, or upper gastrointestinal bleeding. Due to its extreme rarity, all prior studies of giant gastric lipomas have comprised single case reports. The individual case reports are scattered among numerous, and sometimes obscure, journals. This work systematically reviews the literature since 1980, to comprehensively report what is known about this disease and to inform clinicians and clinical researchers what is not known or controversial about this disease.

Research frontiers

A systematic review is important to collate all the prior data presented as case reports to establish what is known about the clinical evaluation (tests) for this disease. This systematic review demonstrates that the standard clinical evaluation should include: (1) abdominopelvic computerized tomography (CT) to demonstrate the characteristic CT findings of a giant gastric lipoma of a well-circumscribed, submucosal, and homogeneous mass with attenuation of fat; and (2) esophagogastroduodenoscopy (EGD) to demonstrate the characteristic endoscopic findings of these lesions of yellowish hue, well-demarcated margins, smooth overlying mucosa, and endoscopic cushion, tenting, or naked fat signs.

This systematic review demonstrates that the following tests are nonstandard or generally obsolete tests: (1) upper gastrointestinal series has been superseded by EGD and should only be performed in highly unusual circumstances; and (2) traditional abdominal ultrasound has been largely superseded by abdominopelvic CT which is a better diagnostic test for this condition, and the traditional abdominal ultrasound should be performed only if the differential diagnosis is broad and not specifically directed at documenting a giant gastric lipoma.

This work systematically reviews several clinically important but controversial topics, including: (1) the role of endoscopic ultrasound: this work shows that conventional mucosal endoscopic biopsies frequently result in a non-diagnostic pathologic diagnosis because giant gastric lipomas are generally submucosal, and therefore endoscopic ultrasound with ultrasound-guided needle biopsies may be necessary if preoperative tissue diagnosis is not obtained by conventional mucosal endoscopic biopsies; and (2) the relative roles of the available therapies: endoscopic mucosal resection, laparoscopic transgastric resection, laparotomy with enucleation, laparotomy with full-thickness wedge resection, and laparotomy with partial gastrectomy and gastric reconstruction.

Innovations and breakthroughs

While several case reports have recently been published on giant gastric lipomas, these case reports generally incorporate limited literature reviews. The present work differs in that it provides a systematic review of the literature. The present work also reports 2 new cases of giant gastric lipomas in a review of 117110 EGDs performed during 11 years at two large teaching hospitals.

Applications

This work provides the following highly clinically relevant conclusions: (1) standard evaluation for suspected giant gastric lipomas should include EGD to demonstrate the characteristic endoscopic findings of yellowish hue, well-demarcated margins, smooth overlying mucosa, and endoscopic cushion, tenting, or naked fat signs; (2) at EGD a submucosal mass that is a suspected lipoma should be biopsied, even though the yield of superficial endoscopic biopsies in pathologically diagnosing a gastric lipoma is relatively low. The diagnostic yield of biopsies at EGD may be increased by using jumbo forceps for the biopsies, or by repeated biopsies at the same site ("well" or biopsy-on-biopsy technique); (3) abdominopelvic CT is a standard test in the evaluation of suspected giant gastric lipomas to demonstrate the characteristic CT findings of a giant gastric lipoma of a well-circumscribed, submucosal, and homogeneous mass with characteristic attenuation of fat; (4) upper gastrointestinal (UGI) series is now generally considered an obsolete test for evaluation of suspected giant gastric lipomas and should be replaced by EGD; (5) conventional abdominal ultrasound is not the preferred test for highly suspected giant gastric lipomas, and should be replaced for this indication by abdominopelvic CT. However, abdominal ultrasound may be a very useful initial imaging test for numerous abdominal conditions in which giant gastric lipoma is in the differential diagnosis; and (6) due to scant data about this rare lesion, and absence of prospective, controlled, therapeutic trials there is no universally accepted standardization of preferred therapies for giant gastric lipomas. Reported therapies include endoscopic mucosal resection, laparoscopic transgastric resection, laparotomy with enucleation, laparotomy with full-thickness wedge resection, and laparotomy with partial gastrectomy and gastric reconstruction. All the reported therapies result in a highly favorable prognosis with no reported mortality among the 32 currently reviewed cases and rare severe morbidity because this tumor is benign, is characteristically biologically nonaggressive, and is well-encapsulated that renders it readily amenable to resection. There is recent interest on selecting less invasive techniques for lesion removal, including endoscopic mucosal resection or laparoscopic removal, as opposed to the traditional laparotomy for removal. This systematic review shows that further research is needed on the optimal therapy for giant gastric lipomas, and on individualizing the therapy according to the clinical presentation.

Terminology

The term giant gastric lipomas refers to gastric lipomas ⩾ 4 cm in diameter. The distinction of size ⩾ 4 cm *vs* size < 4 cm is clinically important because gastric lipomas ⩾ 4 cm generally produce major clinical symptoms from GI obstruction, tumor ulcers, or upper gastrointestinal bleeding, whereas smaller lesions are usually asymptomatic or produce minor symptoms. Furthermore, lesion size often affects the selected therapeutic modality, with lipomas < 4 cm

in diameter often removed endoscopically and lipomas ⩾ 4 cm in diameter generally removed surgically.

Peer-review

This is a very important review paper of the main characteristics of the giant gastric lipomas studied in one Hospital through 10 years of study and follow-up. The diagnosis is very well stablished and also the treatment and prognosis.

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P- Reviewer: Mocellin S, Rodrigo L **S- Editor**: Ma YJ **L- Editor**: A
E- Editor: Huang Y

World Journal of Gastroenterology

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

World J Gastroenterol 2017 August 14; 23(30): 5634-5644

DOI: 10.3748/wjg.v23.i30.5634

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

SYSTEMATIC REVIEWS

Acute colonic pseudo-obstruction: A systematic review of aetiology and mechanisms

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Author contributions: All authors contributed to conception and design of the study; Wells CI acquired and analysed data; all authors contributed to interpretation of data, drafting and revising of the manuscript; all authors have given final approval of the version of the article to be published.

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

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

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Received: May 26, 2017
Peer-review started: May 28, 2017
First decision: June 23, 2017
Revised: June 29, 2017
Accepted: July 22, 2017
Article in press: July 24, 2017
Published online: August 14, 2017

Abstract

AIM

To critically review the literature addressing the definition, epidemiology, aetiology and pathophysiology of acute colonic pseudo-obstruction (ACPO).

METHODS

A systematic search was performed to identify articles investigating the aetiology and pathophysiology of ACPO. A narrative synthesis of the evidence was undertaken.

RESULTS

No consistent approach to the definition or reporting of ACPO has been developed, which has led to overlapping investigation with other conditions. A vast array of risk factors has been identified, supporting a multifactorial aetiology. The pathophysiological mechanisms remain unclear, but are likely related to altered autonomic regulation of colonic motility, in the setting of other predisposing factors.

CONCLUSION

Future research should aim to establish a clear and consistent definition of ACPO, and elucidate the pathophysiological mechanisms leading to altered colonic function. An improved understanding of the aetiology of ACPO may facilitate the development of targeted strategies for its prevention and treatment.

Key words: Acute colonic pseudoobstruction; Acute colonic pseudo-obstruction; Colonic; Intestinal; Pseudo obstruction; Ogilvie's syndrome; Pseudo-Obstruction; Pseudo-Obstruction

Core tip: Acute colonic pseudo-obstruction is associated with considerable morbidity and mortality, though the underlying pathophysiology remains poorly understood.

An abundance of risk factors and associated conditions have been identified, strongly suggesting a multi-factorial origin, and likely culminating in an imbalance in autonomic nervous supply to the colon. Future areas for research are identified and may allow for the development of novel therapeutic or preventative strategies.

Wells CI, O'Grady G, Bissett IP. Acute colonic pseudo-obstruction: A systematic review of aetiology and mechanisms. *World J Gastroenterol* 2017; 23(30): 5634-5644 Available from: URL: http://www.wjgnet.com/1007-9327/full/v23/i30/5634.htm DOI: http://dx.doi.org/10.3748/wjg.v23.i30.5634

INTRODUCTION

Acute colonic pseudo-obstruction (ACPO) is a rare condition characterised by acute colonic dilatation in the absence of mechanical obstruction. It was first described by Sir William Ogilvie in 1948, in two patients with malignant infiltration of the pre-vertebral ganglia[1].

ACPO usually occurs in hospitalised patients with severe illness or trauma, or following general, orthopaedic, neurosurgical, gynaecological or other surgical procedures, with an estimated incidence of 100 cases per 100000 admissions and a mortality rate of 8%[2,3]. Colonic ischaemia or perforation occurs in up to 15%, and is associated with an estimated 40% mortality[4-6]. Therefore, early recognition and appropriate therapy are important determinants of prognosis[2].

The pathophysiology underlying ACPO is poorly understood, with the prevailing hypothesis being an imbalance in colonic autonomic innervation[2,4]. Progress has been limited by a rudimentary understanding of the complex processes regulating colonic motility patterns, the lack of specific animal models for ACPO, and the poor quality of evidence arising from case reports and uncontrolled case series.

An improved understanding of the mechanisms underlying ACPO may aid the development of novel management strategies for this condition. Therefore, the aim of this systematic review was to critically review the literature addressing the definition, epidemiology, risk factors, aetiology and pathophysiology of ACPO with a view to informing knowledge gaps and identifying priority areas for future research.

MATERIALS AND METHODS

A systematic search of the Ovid MEDLINE and Embase databases was performed from inception to 25 May 2017, using the search terms "Ogilvie's syndrome", "pseudoobstruction", and "pseudo obstruction". Google Scholar was also searched using free text entries. Identified articles were screened by title and abstract for inclusion, with subsequent acquisition of full texts. The reference lists of included papers were manually searched, and a hand search of the scientific literature was performed to identify additional relevant publications.

Non-English articles were excluded. There were no exclusion limits by study design; both primary research and review articles were eligible. Full-text articles were evaluated for evidence addressing the definition, epidemiology, risk factors, aetiology and pathogenesis of ACPO. Clinical management has recently been reviewed in depth[7-9], and was beyond the scope of this study, though pathophysiological hypotheses drawn from this evidence were evaluated. A narrative synthesis of the identified evidence was undertaken.

RESULTS

Definitions of ACPO

ACPO is characterised by acute colonic dilatation in the absence of intrinsic mechanical obstruction or extrinsic inflammatory process[10]. ACPO should be distinguished from other acute conditions, such as toxic megacolon, which may have inflammatory or infective causes, as well as chronic intestinal pseudo-obstruction (CIPO) and other causes of megacolon.

Many overlapping terms have been used in the literature to describe ACPO since its original description, reflecting the uncertainly regarding its precise aetiology (Table 1). The term "intestinal pseudo-obstruction" was first proposed by Dudley in 1958[11], with "ACPO" not appearing for another twenty years[4]. Use of "Ogilvie's syndrome" has been discouraged due to ambiguity regarding its meaning[12], though considerable heterogeneity in terminology still exists in recent literature[3,13,14].

Inconsistent terminology has contributed to inconsistency in research and reporting. No standardised clinical definition or diagnostic criteria for ACPO were identified[15], preventing reliable synthesis of data on risk factors or therapeutic strategies[7,8]. Some studies failed to distinguish ACPO from CIPO and other forms of megacolon[10,16], despite these being distinct entities with different mechanisms and therapies[17,18].

Epidemiology

ACPO is uncommon, with an identified incidence of approximately 100 cases per 100000 inpatient admissions[3]. A recent US study reported a declining mortality rate associated with ACPO from 9.4% in 1998 to 6.4% in 2011, although over-diagnosis may have contributed[3]; historically mortality rates were as high as 30%[4]. Colonic perforation and ischemia occur in 10%-20%, with an associated mortality of up to 45%[2,4]. Rates of surgical and endoscopic intervention have declined in recent decades, due to an increasing focus on conservative management and pharmacological therapy with neostigmine[3].

ACPO disproportionately affects elderly and

Table 1 Terms found used to describe acute colonic pseudo-obstruction

Term	Ref.
Large intestine colic	[1]
Ogilvie's syndrome	[4, 153, 155-157]
Pseudo-megacolon	[158]
Adynamic ileus	[159, 160]
Paralytic ileus	[14, 142]
Functional obstruction of the intestinal tract	[161]
Idiopathic large bowel obstruction	[154]
Colonic ileus	[160, 162-164]
Intestinal pseudo-obstruction	[11, 111, 146, 165, 166]
Non-mechanical large bowel obstruction	[167]
Pseudo-obstruction of the large bowel, pseudo-obstruction of the colon	[23, 86, 140, 141, 168, 169]
Acute colonic pseudo-obstruction	[4]
Non-obstructive colonic dilatation	[170, 171]
Malignant ileus	[144]
Cecal ileus	[164]
Acute megacolon	[81, 172]

Table 2 Prevalent medical and surgical risk factors for acute colonic pseudo-obstruction

Category	Risk factors
Surgical	Cardiac surgery, solid organ transplantation, major orthopaedic surgery, spine surgery
Cardiorespiratory	Shock, myocardial infarction, congestive heart failure, chronic obstructive pulmonary disease
Neurological	Dementia, Parkinson's disease, Alzheimer's disease, stroke, spinal cord injury
Metabolic	Electrolyte imbalance, diabetes, renal failure, hepatic failure
Medications	Opiates, anti-Parkinson agents, anticholinergics, antipsychotics, cytotoxic chemotherapy, clonidine
Obstetric/ gynaecological	Caesarean section, normal vaginal delivery, instrumental delivery, preeclampsia, normal pregnancy, pelvic surgery
Infectious	Varicella-zoster virus, herpes virus, cytomegalovirus
Miscellaneous	Major burns/trauma, severe sepsis, idiopathic

comorbid patients; often those with an acute illness on a background of chronic cardiac, respiratory or neurological disease. ACPO also occurs following general, orthopaedic, neurosurgical, cardiothoracic and gynaecological procedures[19]. Vanek *et al*[2] reported 400 cases of ACPO, with approximately 50% resulting from acute medical illness and 50% from post-surgical patients. Incidence rates have been described in spinal or orthopaedic surgery (1%-2%)[13,20], cardiac bypass (up to 5%)[21], and following burn injuries (0.3%)[22]. An abundance of other conditions associated with ACPO were found to have been reported in the literature, the most common of which are summarised (Table 2).

Pathophysiology

Review of the literature revealed a vast array of risk factors for ACPO, supporting a multifactorial aetiology with several pathways leading to a common effect on colonic motor function[23] (Figure 1). However, few published studies were found directly investigating the pathophysiological mechanisms underlying ACPO, with the majority of evidence inferred from basic physiology and other disease states such as post-operative ileus (POI). No animal models of ACPO were identified.

Normal colonic motor activity is regulated at several levels; (1) colonic smooth muscle; (2) pacemaker activity generated by interstitial cells of Cajal (ICC); (3) intrinsic control *via* the enteric nervous system (ENS); (4) prevertebral and spinal reflex arcs; and (5) extrinsic modulation by the autonomic nervous system and hormonal systems. Being a "functional obstruction", ACPO is presumed to result from aberrations in colonic motor activity, though physiological data specifically characterising these abnormalities was not identified.

Autonomic imbalance

Altered extrinsic regulation of colonic function by the sympathetic and parasympathetic nervous systems is the most commonly suggested mechanism for ACPO[10,24-29]. This mechanism was first postulated by Ogilvie, who proposed a "sympathetic deprivation" of the colon[1]. Current theory favours a relative excess of sympathetic over parasympathetic tone, though it is unclear whether this is due to increased sympathetic activity, reduced parasympathetic signalling, both, or either. The prevailing hypothesis remains that ACPO is the result of reduced parasympathetic innervation to the distal colon, leading to an atonic segment and functional obstruction[2,10,27,28,30-32]; however no published physiological data was found to directly support or refute this claim[28,29].

The colon receives sympathetic innervation from the prevertebral ganglia (proximally *via* the superior mesenteric ganglion; distally *via* the inferior mesenteric ganglion). Nerve fibres follow the respective arterial routes[33], and the proximal colon appears to have a richer sympathetic innervation[23]. The parasympathetic supply to the mid-gut is *via* the vagus nerve while the hind-gut is innervated by sacral parasympathetic outflow (S2-4)[33], although animal studies have demonstrated some overlap between these distributions[34]. The transition point from distended to normal colon in ACPO usually occurs near the splenic flexure[2,10,35], where there is a transition in the innervation for both sympathetic and parasympathetic supply to the colon, supporting autonomic imbalance as a key step in pathogenesis.

Most patients with ACPO are unwell with major illness, therefore having increased systemic sympathetic drive[23,36,37], potentially contributing to autonomic imbalance at the level of the colon. Other reported cases have involved pathology potentially disrupting the autonomic supply to the colon, such as retroperitoneal tumours or haemorrhage[38,39]. Interestingly, ACPO cannot be reproduced in humans or animals by splanchnic or pelvic nerve transection[40,41], thus the pathogenesis appears more complex than excess or

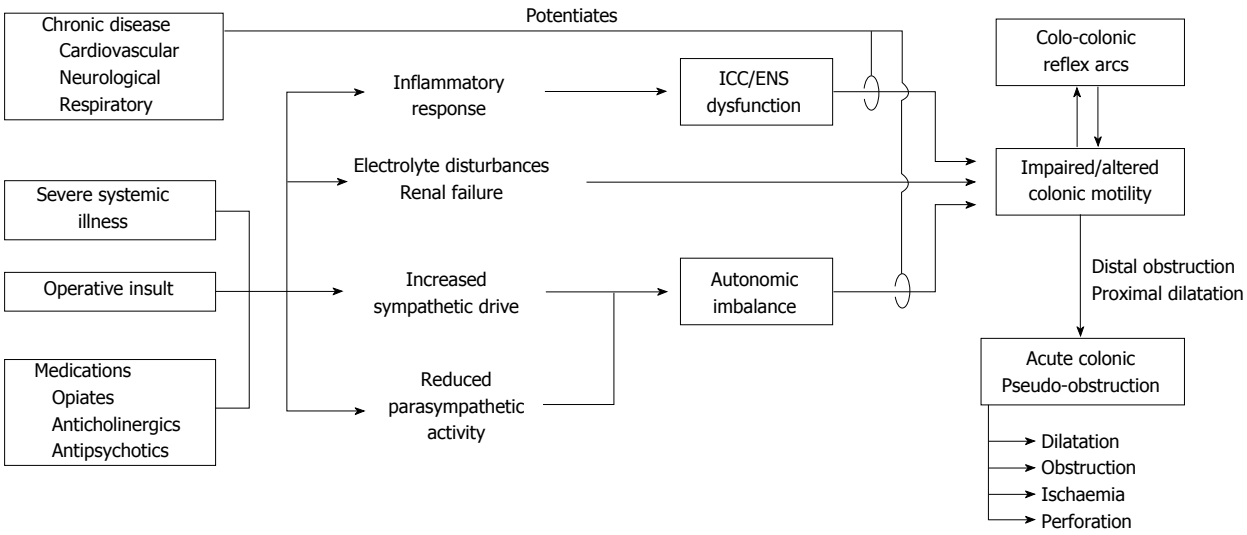

Figure 1 Pathophysiological factors that have been implicated in the development of acute colonic pseudo-obstruction. ICC: Interstitial cells of Cajal; ENS: Enteric nervous system.

deficiency of sympathetic or parasympathetic activity alone.

Neostigmine is an acetylcholinesterase inhibitor and parasympathomimetic commonly used in management of ACPO, further supporting autonomic imbalance as a key pathophysiological mechanism. Early studies investigated use of guanethidine, a sympatholytic, followed by neostigmine[42]. Guanethidine was later shown ineffective[43], while neostigmine has been proven efficacious in several randomised trials[7,44-46]. Prior studies have shown that parasympathetic stimulation with neostigmine triggers colonic high amplitude propagating sequences (HAPS) in both dogs and humans[47,48], which has been proposed as a possible mechanism for its decompressive effect in the functionally obstructed colon[49]. However, HAPS primarily arise in the proximal colon, and it is unknown what effect neostigmine has on other prominent colonic motor activity such as cyclic events, or the role of these events in ACPO[50].

Colonic reflex arcs

Several spinal and ganglionic reflex arcs are involved in regulating intestinal motor function. The colo-colonic inhibitory reflex refers to inhibition of proximal colonic motor activity in response to distal colonic distention[51,52]. Conversely, proximal distension also causes a reduction in basal intraluminal pressure in the distal colon[51]. Evidence from animal models suggests these reflex arcs are mediated *via* afferent mechanoreceptors synapsing with adrenergic efferent neurons in the prevertebral ganglia and spinal cord[18,51,52].

These reflexes provide a possible mechanism to explain how disordered motility and distension of one colonic region may potentiate dilatation of other regions, contributing to ACPO[18,26,28,30]. Some authors have pointed to the therapeutic success of epidural anaesthesia and splanchnic nerve block in case reports as evidence for this mechanism[10,53], though it is difficult to ascertain whether this is due to disruption of the efferent limb of these reflex arcs, or simply reduction in the extrinsic sympathetic supply to the colon. Furthermore, epidural anaesthesia has been implicated as both a cause of and a therapy for ACPO[18,53,54]. The lack of high-quality evidence identified in this area makes it difficult to determine the effects of spinal or ganglionic blockade in ACPO and the precise role of the colo-colonic inhibitory reflex.

Intrinsic colonic dysfunction

ICC are the pacemaker responsible for generating electrical slow waves, which are modulated by the ENS, resulting in the rhythmic contractile activity of the intestine[55,56]. Despite their recognised importance in the control of gastrointestinal motility, few studies were identified that specifically investigated the roles of enteric neurons or ICC in ACPO.

Permanent impairment of the ENS, ICC, and/or myopathy characterises many forms of CIPO[24,57-59]. However, the acute onset, reversibility, and different epidemiology of ACPO implies a distinct patho-physiological process from CIPO, hence these findings should not be extrapolated to the acute form of pseudo-obstruction.

Jain *et al*[57] investigated ICC using immuno-histochemistry in patients with pseudo-obstruction syndromes, showing two patients with ACPO had a normal number and distribution of ICC. However, whether ICC function is affected in ACPO remains unknown. Choi *et al*[35] reported a reduction and degeneration of enteric ganglionic cells in the resected colonic specimens of four patients with pseudo-obstruction, but it is unclear whether these patients had ACPO or CIPO, and whether these histological abnormalities represent cause or effect of the colonic

dilatation and pseudo-obstruction. Without further pathophysiological studies, the role of the ENS and ICC in the development of ACPO remains unknown.

Nitric oxide (NO) is an important inhibitory neurotransmitter released by colonic enteric neurons, and has been implicated in colonic dilatation and dysfunction in toxic megacolon[37,60,61] and colitis[62]. Several authors have speculated whether NO may also have a role in ACPO, however no physiological evidence was found to support this claim. However, polyethylene glycol (PEG), an osmotic laxative that may also reduce NO production[63,64], has been shown to reduce relapse rates after decompression in ACPO[65]. This finding could be explained by either the laxative effects of PEG or its effect on nitrinergic signalling. A number of novel pharmacological treatments targeting NO regulation have recently been discovered, though no studies were identified that applied these therapies in patients with ACPO[66].

Chronic disease and pharmacological factors

Notably, many ACPO patients are elderly and have chronic cardiac, respiratory or neurological disease, with onset often occurring in the context of a further acute physiological insult[2,3]. Chronic stress conditioning, similar to the effects of chronic disease, potentiates excitatory and inhibitory neurotransmission[67], potentially explaining these associations. The ENS and its extrinsic regulation are affected in several conditions commonly associated with ACPO, including diabetes mellitus[68,69], Parkinson's[70,71] and Alzheimer's disease[70,72,73]. Furthermore, both the ENS and ICC have been shown to degenerate with age[74-76], which may partly account for the elderly preponderance of this disorder[2,3,29,77].

Patients with chronic conditions are also more likely to be on multiple medications affecting colonic motility[78,79], and may therefore be predisposed to the development of ACPO. Numerous medications have been associated with ACPO, including anticholinergics, opiates, calcium channel blockers, and psychotropic drugs[80-82]. Furthermore, use of anti-motility agents is predictive of poor response to neostigmine[83], while methylnaltrexone has been successfully used in a patient with ACPO who failed to respond to two doses of neostigmine[84].

Many medications associated with ACPO modulate the autonomic nervous system, supporting disruption of these pathways as a key pathophysiological mechanism. Clonidine[18,85-87] and amitraz[88] are α_2-adrenergic agonists that have been associated with ACPO. At the level of the colon, α_2-adrenergic signalling reduces release of acetylcholine from enteric neurons, resulting in a relative imbalance of sympathetic over parasympathetic supply[89,90], consistent with current theory regarding the pathophysiology of ACPO.

Obstetric causes

The operation most commonly resulting in ACPO is caesarean section[2,26,91-99], but ACPO also occurs after normal and instrumental vaginal delivery[100-102]. A recent systematic review identified that preeclampsia, multiple pregnancy, and antepartum haemorrhage/placenta praevia appear to be more common in women who develop ACPO following caesarean section[103]. However, it remains unclear how these events result in acutely perturbed colonic function. Some authors have proposed that compression of parasympathetic plexuses by the gravid uterus may contribute[26,27,104], while others have hypothesised that the uterus may fall back into the pelvis following delivery, causing a mechanical obstruction at the rectosigmoid colon[36,105].

Pregnancy is also associated with high levels of progesterone and glucagon, both of which have been shown to diminish the tone of the large bowel[92], and may predispose to ACPO when combined with acute physiological disturbances such as caesarean section, preeclampsia, peripartum sepsis, or haemorrhage. Resting sympathetic vasomotor outflow is increased during the third trimester, even in women with normal blood pressure[106,107], while autonomic dysfunction and sympathetic overactivity are a feature of severe preeclampsia[108,109]. It is unknown whether sympathetic supply to the colon is also affected in these states, but this could plausibly contribute to autonomic imbalance and ACPO. Finally, prostaglandins are intimately involved in parturition[110], and have also been suggested as a possible contributor to ACPO in obstetric patients[92] (*discussed in next section*).

Metabolic factors

A disrupted "*milieu interieur*" is common in ACPO, which may precipitate or exacerbate the effects of altered autonomic function or other mechanisms. A host of metabolic factors have been shown to influence colonic motility, though few studies have investigated these specifically in ACPO. However, the isolated colonic dysfunction characterising ACPO makes it unlikely that metabolic abnormalities are the sole pathophysiological explanation.

Renal failure and electrolyte disturbances often accompany ACPO, although it has been disputed whether this is a cause or effect of the pseudo-obstruction[4,16,32,39,83,111]. Alterations in potassium and other electrolyte concentrations affect ion channel function and may alter ICC pacemaker or smooth muscle activity[112,113]. Furthermore, electrolyte imbalance has been identified as a predictor of a poor clinical response to neostigmine[83].

Prostaglandins and cytokines are both well-established inflammatory mediators of gastrointestinal motility[114,115]. Pro-inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-1β have implicated in POI[114,115], but have not yet been investigated in ACPO. Prostaglandins have been implicated in POI[115,116], CIPO[117,118], acute small intestinal dysmotility[119], and also affect ICC function and slow wave frequency[120]. However, no studies were identified investigating the

role of prostaglandins in the development of ACPO. Cyclooxygenase-2 (COX-2) expression is increased in the distended colon of mice with experimentally-induced mechanical obstruction, and it has been suggested that this is responsible for reduced motility *via* the effects of prostaglandin E2[121]. However, caution must be taken when extrapolating these results to humans and to ACPO[122].

Viral enteroneuropathy

ACPO has also been associated with several viral infections, most commonly herpes zoster reactivation in low thoracic or lumbar distributions[123-130], but also disseminated varicella zoster[131-134], acute cytomegalovirus[135], and severe dengue[136].

Several mechanisms have been proposed to explain these associations, all involving autonomic dysfunction. Reactivation of herpes virus in enteric ganglia may result in a sympathetic autonomic neuritis[123,124,137,138], presumably diminishing colonic sympathetic supply. Local segmental colonic inflammation with afferent stimuli to the sacral nerve roots and blockage of colonic parasympathetic supply has also been suggested[138], while other authors have hypothesised that viral spread from the dorsal root ganglia to the thoracolumbar or sacral lateral columns may interrupt sacral parasympathetic pathways[137]. Involvement and inflammation of the intrinsic enteric nervous system, and post-viral dysautonomia have also been suggested[136,137,139].

Other hypotheses

Several alternate hypotheses regarding the pathophysiology of ACPO were identified. Compromised vascular supply to the colon was suggested in many early reports[11,140,141], but now thought to represent an exacerbating complication rather than a cause. "Hinge-kinking" of the colon at the transition from retro- to intra-peritoneal structures has also been proposed[142], however, intra-operative and radiological findings generally suggest a "gradual" transition in colonic calibre[23]. Other identified hypotheses lacking supporting evidence included the "air-fluid lock syndrome"[143-145], and colonic distension due to aerophagy in chronic respiratory disease[146,147].

DISCUSSION

Despite considerable speculation, this comprehensive and systematic review of the literature finds that the mechanisms underpinning ACPO still remain uncertain. Limited physiological evidence from patients with ACPO was found to exist, and the lack of an animal model has required extrapolation of data from other disease states. The abundance of identified risk factors and associated conditions supports a multifactorial hypothesis, with aberrations in function at any of several levels seemingly able to lead to common or similar effects on the colon.

Overall, autonomic imbalance is the most strongly supported pathophysiological contributor, and likely plays a key role in the development of ACPO. Evidence supporting a major role for autonomic imbalance includes the observation that most patients with ACPO are systemically unwell, or have other pathology disrupting the autonomic supply to the colon. Furthermore, the transition point from distended to normal colon in ACPO usually occurs at the splenic flexure, corresponding to the change in autonomic innervation of the mid-gut and hind-gut. However, the precise aberrations in autonomic function leading to ACPO remain unclear.

ACPO is difficult to rigorously investigate due to its acute and sporadic nature and heterogeneity. The plethora of case reports identified in the literature is testament to the large number of illnesses associated with this condition. It is also clear that a categorical clinical definition of ACPO is needed to standardise research, and improve the quality of published evidence. Distinct subtypes of ACPO have recently been proposed according to gut wall thickness on cross-sectional imaging[148], and further work should investigate the aetiology and prognostic significance of this phenomenon.

Importantly, the precise aberrations in colonic motor activity underlying ACPO have not been characterised. Despite the common assumption that the distal colon is atonic, direct physiological evidence supporting this claim could not be identified, and it remains unknown whether ACPO is fundamentally due to atony, dysmotility, or spasm in part or all of the colon, or is due to heterogeneous motility states. Furthermore, although many authors have argued that the sympathetic system decreases colonic motility and the parasympathetic increases contractility[10,18,28,30,43], this is now recognised as overly simplistic, with further layers of modulation within the ENS, ICC and smooth muscle[27,33]. It is also important to note that the therapeutic success of neostigmine also does not specifically prove interrupted parasympathetic supply to the colon as the sole pathophysiological mechanism underlying ACPO[29], despite this being suggested by many authors[2,42,43,149,150]. Indeed, provocation of HAPS and colonic emptying by neostigmine may be sufficient to overcome an alternative pathophysiological mechanism and result in decompression of the functionally obstructed colon.

High-resolution colonic manometry has recently been used to characterise the abnormalities underlying colonic dysmotility in the early post-operative period, demonstrating increased cyclic motor activity in the left colon, rather than atony, and an absence of HAPS[151,152]. Applying similar research methods to ACPO would be a particularly interesting research avenue, with potential to rigorously define the altered motility patterns underlying the disorder. It is interesting to note that transient "ring[s] of spasm" were noted intra-operatively in the distal colon of several of the original

descriptions of this syndrome[1,153,154], though the pathophysiological significance of these remains unclear. Other areas for future pathophysiological investigation include the development of an animal model of ACPO, histological examination of resected specimens, and rigorous investigation of the impact of preventative or therapeutic strategies on colonic function.

This systematic review and appraisal of the literature surrounding ACPO has identified limited evidence investigating the mechanisms underlying this condition. A number of areas for further study have been identified. It is hoped that an improved understanding of the pathophysiology of ACPO will lead to the development of targeted strategies for its prevention and treatment.

COMMENTS

Background

Acute colonic pseudo-obstruction (ACPO) is an uncommon condition, characterised by acute colonic dilatation in the absence of a mechanical obstruction. Colonic ischaemia or perforation may occur in up to 15% of patients, and is associated with considerable morbidity and mortality.

Research frontiers

The pathophysiology underlying ACPO is poorly understood, with a plethora of associated conditions reported in the literature. The prevailing hypothesis is an imbalance in colonic autonomic innervation, though few articles have thoroughly examined the evidence supporting this claim.

Innovations and breakthroughs

This systematic review finds that the mechanisms underpinning ACPO still remain uncertain. Autonomic imbalance is the most strongly supported pathophysiological factor, though the precise aberrations leading to ACPO are unclear. A number of other factors may contribute to ACPO, including colonic reflex arcs, intrinsic colonic dysfunction, chronic disease, and pharmacological or metabolic disturbances.

Applications

A number of areas for future investigation have been identified, including the use of high-resolution colonic manometry to rigorously define the altered motility patterns underlying ACPO, the development of an animal model, and histological examination of resected specimens. An improved understanding of the pathophysiology of ACPO may lead to the development of novel preventative or therapeutic strategies.

Terminology

Pseudo-obstruction is characterised by a clinical presentation suggestive of intestinal obstruction, in the absence of a mechanically obstructing lesion. ACPO is the acute form of colonic pseudo-obstruction, while chronic intestinal pseudo-obstruction is a distinct entity with different mechanisms and therapies. "Ogilvie's syndrome" has also been used to refer to ACPO, though this term is now discouraged due to ambiguity regarding its meaning.

Peer-review

This is a very interesting review regarding the etiology of acute colonic pseudo obstruction. The author introduced a number of articles regarding this issue.

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P- Reviewer: Koda K **S- Editor**: Qi Y **L- Editor**: A
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ISSN 1007-9327